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PLASMOCYTIC XANTHOGRANULOMA

By

HARRY BROCKS and VIGGO ESKELUND

Received 20.1.61

The lipidoses make up an extremely variegated group genetically as well as clinically. A rough classification is based mainly on the nature of the lipids.

A sub group the xanthomatoses is characterised by an accumulation of cholesterol and its esters in different tissues. Their classification varies somewhat and is still marked by some uncertainty. The one used by *Cavernagh* (1956) is as follows

1. *Xanthomatosis associated with raised serum lipids*
   a. Essential hypercholesterolaemic xanthomatosis
   b. Symptomatic hypercholesterolaemic xanthomatosis
   c. Essential hyperlipaemic xanthomatosis
   d. Symptomatic hyperlipaemic xanthomatosis
2. *Xanthomatosis with essentially normal serum lipids*
   a. Hand Schuller Christian disease
      Eosinophilic granuloma
      Letterer Siwe disease
   b. Local xanthomatous conditions of reactive type

In other words a number of widely different diseases whose common feature is accumulation of cholesterol but even this is not always present.

The classical triad of Hand Schuller Christian s disease cranial defects exophthalmos and diabetes insipidus often occurs in an ailment which comprises apart from other localisations other phases such as Letterer Siwe disease and eosinophilic granuloma Letterer S...

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1 AC

from the xanthomatosis found in Hand-Schüller-Christian's disease. Nevertheless, they are interpreted as different phases of the same basic disease. Transitional forms between the different phases have been encountered. *Wallgren* (1940) found accumulation of cholesterol in reticulum cells in Letterer-Siwe's disease which he considered an initial stage of rapid course, generally fatal before xanthomatous reticulosis had time to manifest itself. Eosinophilic granuloma was originally interpreted as a disease *sui generis* (*Lichtenstein & Jaffe* 1940), but very soon it was suggested that it might be closely related to Hand-Schüller-Christian's disease (*Farber, Green & Farber* 1942, *Ahlström & Welin* 1943).

Finally, in 1944, it was established by *Engelbreth-Holm, Teilum & Christensen* that the disease was a phase of Hand-Schüller-Christian's disease. These authors had found the histological course to be in four phases:

1. A hyperplastic, proliferative, reticulo-histiocytic phase.
2. A granulomatous phase characterised by eosinophilic cells, macrophages, and giant cells.
3. A xanthomatous phase.
4. A phase of fibrous healing.

Transitional forms between Letterer-Siwe's disease, eosinophilic granuloma, and Hand-Schüller-Christian's disease have subsequently been reported by *Beskow* (1946), *Berg* (1946), *Bartels* (1947), and *Poul Bjerre Hansen* (1949).

While Hand-Schüller-Christian's disease in *all* its phases is clinically and pathologically rather well known, another, more common form of xanthomatosis, xantho-granuloma, is still little known and a rather vague conception.

Oberling (1935) was the first to call attention to this disease. He published three cases observed by himself, supplemented by three from the literature, presumably of the same nature. The disease was localised to the "retroperitoneal space", and showed a rather varied clinical course. In some cases it appeared as a benign, well-circumscribed lesion, in others as an invasive tumour with a tendency to local recurrence and metastasizing to various organs (liver, lungs, heart, and serous membranes). The tissue was extremely varied. Its consistency was firm or soft, and the cut surface would be characterised chiefly by sulphur-yellow patches and strands of cholesterol deposits. The microscopic appearance was also motley. There would be cellular and vascular areas, in some cases fairly uniform, suggestive of sarcoma, and in others more like a chronic or subacute inflammation with infiltrations of lymphocytes, histiocytes, plasma cells, giant cells of the myeloplaque type, polymorphonuclear leucocytes, and foam cells. The "inflammatory" areas showed transitions to acellular and avascular strands looking most of all like cicatricial tissue. Necrosis was rare and eosinophilic

TABLE 1

Twelve Cases of Xanthogranuloma from the Literature

Case No	Age Sex	Author	Site of tumour	Other tumours	Follow up
1	31♂	Oberling	R iliac fossa	None	Recurr tumour removed 2 yr postop
2	25♂	Oberling	Ant to r kidney	None	Well 3 months postop
3	31♀	Oberling	R side of abdomen	None	None
4	29♀	Dietrich	Retroperitoneum	Xanthomatosis liver heart, retrobulb	Autopsy
5	33♀	Nothen	Retroperit l side	Spleen, liver lungs	Autopsy
6	45♂	Noel & Michel Béchet	L pararenal area scrotum	Scrotum	Recurr in 4 yr died 3 mo postop
7	60♂	Ackerman	Retroperitoneum	None	None
8	54♂	Melicow	Retroperit kidney	Diffuse xanthomatosis	Died
9	51♂	Melicow	Subphrenic space to pelvis	None	None
10	44♀	Melicow	Below hepatic flexure	None	None
11	63♂	Melicow	Retroperitoneum	None	Died
12	51♀	Waller, Hellwig and Barbosa	Retroperitoneum	Visceral eosinophilic granuloma	Well 15 months postop

cells were scattered and sparse The literature from 1935 to 1937 contains only 12 cases of this disease (cf Table 1) all of which appear to have been related to "the retroperitoneal space" Secondary deposits in other organs have been found in four cases The case reported below has certain features in common with Oberling's xanthogranuloma although it differs in several respects, clinically as well as histologically

CASE REPORT

The patient was a woman, agred 70, who had never been seriously ill There was no family history of diseases resembling the one to be described May 2 1951 she had been admitted to a medical ward (Hjorring Central Hospital case rec 782 51) with swelling and tenderness of the cervical lymph nodes Three or four years previously she had suffered from an acute febrile condition diagnosed as epidemic parotitis A swelling persisted on the right side of the neck and remained unchanged until 3 or 4 months prior to admission when it started growing steadily in size and firmness This was accompanied by pain radiating towards the right ear and some swelling on the right side of the neck During the 3 weeks prior to admission the patient had suffered from a cold in the head with rhinitis and cough and felt very tired No night sweats itching cardiac or pulmonary symptoms

On admission there was moderate tenderness and enlargement of both submaxillary glands, especially the right one, and of the right parotid gland The consistency was hard as stone and the surface rough slightly nodular The skin was movable against the underlying structures and there was no swelling of the lacrimal glands A slight enlargement of the axillary lymph nodes was found A few days later it had been recorded that the tongue was moist naked with slight papillary atrophy Fissures in both corners of the mouth Enlargement of both parotid glands and large firm lymph nodes in both submaxillary regions Among the results of other tests it may be mentioned that the Hb level on admission was 76 per cent varying up to 87 per cent and on discharge 66 per cent The E S R fluctuated from 94 on admission to 64 about 4 weeks later On discharge it was 91 The blood pressure on admission was 165/95 decreasing to 135/80 on discharge The red blood count was 4 2 million on admission and evenly declined to 3 2 mill on discharge The white count was 4 300 on admission steadily increasing to 8 800 and then fell somewhat The colour index fluctuated from 0 92 to 1 05 being 0 98 on discharge Differential count on admission showed rod shapes and segm neutrophils to be 55 per cent increasing to 77 per cent on discharge Eosinophils were 4 per cent on admission one week later 14 per cent and on discharge 3 per cent having fluctuated between 2 14 per cent The lymphocyte count varied from 18 to 34 per cent Monocytes also fluctuated somewhat from 9 per cent on admission to 2 per cent on discharge Occasionally a plasma cell was found in the blood Wassermann reaction negative E C G showed no abnormalities Mantoux (1/10 mg) gave a reaction measuring 10 × 9 mm Gastric lavage did not show growth of acid fast rods The urine did not contain protein or sugar Chest radiography revealed a few calcific patches as large as peas *in the right central field In addition slightly increased* lung markings and sligt blurrings basally on the left Sinus and diaphragm free Diagnosis Calcifications infiltrations of left lung (seq?) Repeated X rays 12 days later no longer showed definite changes in the area on a level with the cardiac apex apart from slightly increased partially transverse strands Diagnosis No definite *abnormality A later* X ray investigation (on May 21) including a lateral film showed a trace of enlarged lymph nodes in the hilar regions

Sternal aspiration on May 17 showed the following distribution Segmental 22 per cent rod shapes 8 per cent metamyelocytes 26 per cent myelocytes 8 per cent promyelocytes 5 per cent eosinophils 1 2 per cent lymphocytes 12 per cent plasma cells 1 per cent orthochromatic 6 per cent polychromatic 5 per cent basophils 2 per cent reticulum cells 2 per cent Conclusion Slight myeloid hyperplasia of a type like that seen in infections (*H Gormsen*)

On May 8 the right submaxillary gland was removed It was the size of a walnut and very firm The patient was treated with streptomycin and PAS but this medica

tion had to be abandoned after a little more than 3 weeks because of nausea She also had 2 X ray treatments and was feeling well on discharge

Reactions for toxoplasmosis and histoplasmosis were negative Antistreptolysin titre 1400–450 After discharge the F S R and antistreptolysin titre remained somewhat elevated Repeated examination of the sternal marrow on Aug 20 1951 showed the following myelocytes 1 per cent metamyelocytes 11 per cent rod shapes 20 2/3 per cent segmental 10 1/3 per cent eosinophilic myelocytes 1 1/3 per cent eosinophilic leucocytes 5 per cent lymphocytes 17 1 3 per cent, plasma cells 2 1/3 per cent reticulum cells 2 1/3 per cent, shadow cells 7 per cent basophilic normoblasts 3 2 3 per cent polychromic 1 1 3 per cent orthochromic 8 2/3 per cent Conclusion Sternal aspirate with slight eosinophilia and some left shift of the granulocytes (*Eskelund*)

The tissue from the submaxillary region measured, after fixation, 3 × 2 × 3 cm The surface and cut surface were white and the consistency firm

Microscopic examination showed a solid, very polymorphous tissue, in some places sharply demarcated from the submaxillary gland, while in other places it infiltrated the glandular tissue without destroying it In the marginal zone the tissue was cellular, consisting predominantly of histiocytic cells with round or oval nuclei, fairly rich in chromatin and uniform in size The nucleoli were small The cell borders were very blurred and the cytoplasm was pale and slightly eosinophilic with delicate fibrils (Fig 1) Interspersed between these cells there was a certain number of plasma cells and some lymphocytes, lying singly or in clusters The tissue was vascular, the vessels being mainly of capillary nature There was also an ample, delicate network of reticulin and a more sparse accumulation of collagen fibrils (Fig 2) In the denser lymphotic infiltrations the reticulin was sparse In the more central areas of the infiltrating tissue, the picture was more varied In some places the tissue was rather loose, made up of histiocytes, generally larger than in the marginal zone Their nuclei were round or oval, larger and more varied than the nuclei of the cells in the marginal zone The cells were rounded, polygonal, or spindle-shaped, forming ill-defined [illegible] lasm was ample, pale, [illegible] in some places deli- [illegible] defined in many places Here and there the cells were rather scattered The nuclei contained less chromatin than the nuclei in the marginal zone and often they were vacuolated Some cells had two nuclei (Fig 3) The nuclear membrane was distinct and there was one or two nucleoli The vessels were not as dense as in the marginal zone They were immature, of low differentiation, often merely endothelial tubes There was an ample network of delicate reticulin fibres, often framing individual cells whose cytoplasm was argentophilic Only a few collagen fibrils were present

From these fairly uniform areas, there was a transit[illegible] varied area[illegible]

[illegible]

[illegible] present in dense clusters The

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plasma cells varied considerably in size and shape there were rounded oval spindle shaped and angular cells with a pyroninophilic cytoplasm Moreover there was a number of small pyroninophilic cell fragments The nuclei of the plasma cells varied a good deal in size and some of the cells had two nuclei In most of the cells the chromatin was rather coarse of the typical peripheral granular arrangement but there were also especially in small cells nuclei which were of a denser structure and without coarse granules Such cells had rather sparse cytoplasm Frequently the nuclei were not eccentrically arranged and there was no paranuclear vacuolation In several places there were plasma cells often of angular shape in rows along the capillaries just outside the endothelium (Fig 4) Lastly there were in several places Russell bodies of varying size among plasma cells Lymphocytes were also in part scattered among the plasma cells and in part arranged in dense clusters In some places there were small reaction centres

The tissue also contained connective tissue cells of varying degree of maturity and collagenous fibrils partly in the form of a fairly diffuse infiltration and partly as somewhat denser ill defined strands of varying width In these strands some of the collagenous fibrils were coarse and hyalinized No major acellular fibrous areas were seen In the tissue there was also a dense network of argentophil fibrils especially in the plasmocytic infiltrations and among the collagenous fibrils A somewhat smaller number of fibrils were encountered in areas where histiocytic cells and lymphocytes predominated In a few places there were also clusters of foam cells (Fig 5) Lastly there were scattered small clusters of granules giving a positive iron reaction No lipid staining was performed

Histological diagnosis

Salivary gland tissue with specific looking chronic inflammatory changes The possibility of an inclusion disease was considered as the histiocytes contained in a few places rounded bodies varying up to 5 6 mm in diameter Often they were surrounded by a pale zone of somewhat varying width

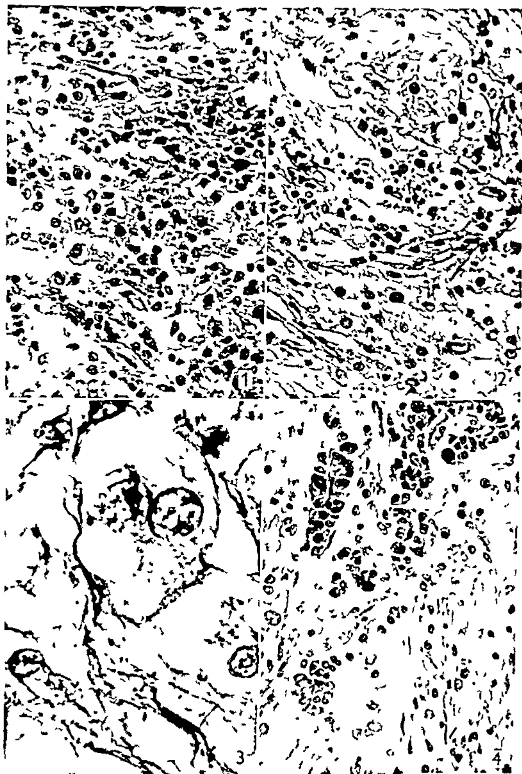
During the subsequent years the patient was in good health but the F S R and antistreptolysin titre remained somewhat raised

On June 21 1956 she was admitted to the surgical ward because of

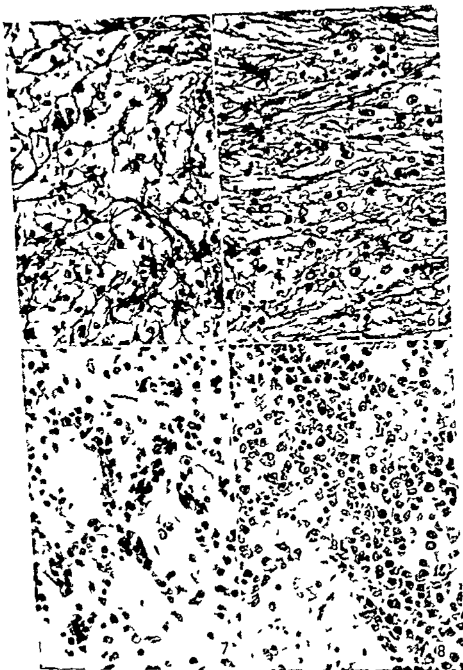
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Figs 1 4

Fig 1 Histiocytic cells from the submaxillary region H E staining (285 ×)
Fig 2 Histiocytic cells from the submaxillary region V Gieson staining (285 ×)
Fig 3 Large histiocytic cells with reticulin fibres Foot staining (1080 ×)
Fig 4 Histiocytic cells and plasma cells ensheathing capillaries H E staining (285 ×)



Figs 1 4



Figs 5 8

pain in the right flank radiating into the back and down the right side of the abdomen She stated that during the past year she had been having periodical attacks of this nature On admission she was found to be rather pale In the left submaxillary region there was one firm lymph node, the size of a hazel nut Below the right costal border there was a vague swelling

There was no splenomegaly and no sign of ascites E S R 132, and B P 140 80 The urine contained no protein or sugar, and microscopic examination showed no abnormality Blood urea 42 mg per cent, icteric index 4, thymol reaction negative, alkaline phosphatase 7

Blood study Hb 69 per cent, R B C 3 1 mill, W B C 7,900, differential count Lymphocytes 18 per cent, myelocytes 1 per cent, eosinophilic leucocytes 2 per cent, monocytes 5 per cent, rod shapes 7 per cent, segmental 67 per cent Wasserman and gonoc reactions negative, E C G normal Diastase 150 (4 ×), bilirubin —, urobilin +, +, +, (+) Takata + + + Widal + *No salmonella typhi O antigen, + paratyphi* B H antigen in dilution 1/50 No paratyphi O antigen No Brucella abortus (*Bang*)

Pyelography revealed changes indicating a tumour in the right kidney On July 4, operation was performed under Pento-Curacit – N O anaesthesia The approach was through a right-sided lumbar incision There was no difficulty in mobilizing the kidney There was a tumour *in the central area A forceps was applied to the pedicle, the kidney* was removed and the pedicle ligated The ureter was intersected rather low The patient made an uncomplicated recovery

The kidney measured about 11 × 6 × 5 cm In the pelvic region, extending into the lower pole, there was a tumour-like infiltration with a rather firm marginal zone and a softer consistency towards the centre In places the firm, whitish tissue showed a somewhat yellow hue

Microscopical examination showed that the tumour-like tissue infiltrated the renal tissue, the pelvis and the perirenal tissue It varied somewhat in appearance, and there were several varieties of cells In some places the tissue was rather loose, consisting mainly of large, irregular, often angular histiocytic cells with a loose, finely granular or somewhat fibrillar cytoplasm and round or oval nuclei fairly poor in chromatin but varying considerably in size (Fig 6) The nucleoli were relatively large In places, the cytoplasm contained lymphocytes or small granular inclusions In several places the cell borders were frayed

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Figs 5 8

Fig 5 Foam cells and dark histiocytes with ample reticulin Foot staining (285 ×

Fig 6 Tissue from the kidney showing histiocytic cells Ample reticulin Foot staining (285 ×)

Fig 7 Plasma cells around capillaries *H E* staining (285 ×)

Fig 8 Clusters of plasma cells of varying shape and size Unna Pappenheim staining (285 ×)

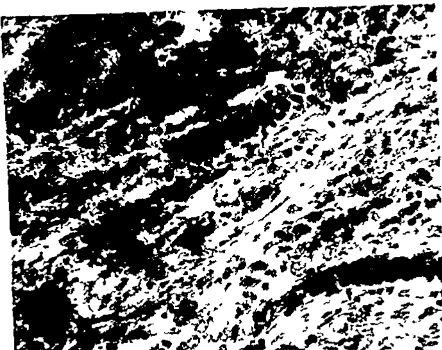


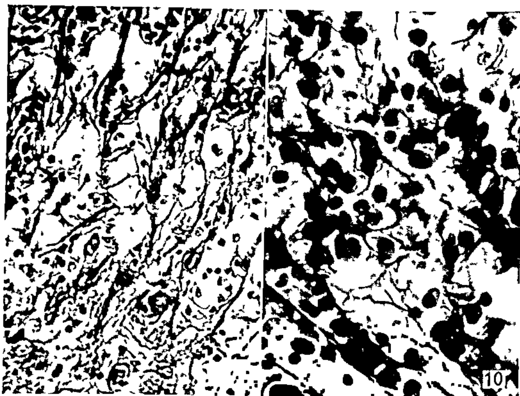
Fig 11

ample quantities of lipid, in some places as dense extracellular accumulations and in other places as histiocytic inclusions These cells were on the whole slightly smaller than those without a lipid content In some of the cells the lipid was in the form of scattered, rather coarse granules in the cytoplasm, while in others it was confluent Some of the cells containing lipid looked like plasma cells in size and shape The nuclei were most frequently excentrically arranged but dense in structure and rich in chromatin Furthermore, there were here and there plasma cells whose cytoplasm was of a faintly yellow hue Scattered in the tissue there were small, yellowish brown granular accumulations giving a positive iron reaction

PAS staining showed that some of the large irregular histiocytic cells had a reddish cytoplasm Unna Pappenheim staining revealed the reaction t [illegible]
there [illegible]

Thi [illegible]
found at the former operation, infiltrated the renal tissue without having caused degenerative changes of the parenchyma There was hyalinization of a few glomeruli, but probably not more than normal for the patient's age

The histological examination including lipid staining left no doubt that this was a case of xanthomatosis And indeed, chemical analysis



Figs 9–10

Fig 9 Foam cells in a stroma showing ample reticulin Foot staining (285 ×)
Fig 10 Infiltration with plasma cells showing reticulin Foot staining (420 ×)

and blurred From these areas of fairly uniform structure there was a gradual transition to others, with major or minor accumulations of plasma cells In some places the plasma cells formed a kind of sheath around the capillaries (Fig 7), while in other places they lay scattered or in clusters in the tissue They varied somewhat in size and shape, but the nuclei showed a fairly uniform chromatin content several cells had two nuclei (Fig 8) Among the clusters of the plasma cells there was also a scattered, sparse infiltration of lymphocytes and in places eosinophilic leucocytes In other sites the tissue was somewhat denser with delicate or coarser collagen-fibrils In these sites, too, there were considerable accumulations of plasma cells and also fairly large lymphocytic infiltrations In the connective tissue, in several places, typical foam cells lay scattered or in denser clusters Among the plasma cells a number of Russell bodies were observed Silver staining showed the large irregular histiocytic cells to be slightly argentophilic Foot staining revealed, around these cells, a coarse or delicate network of argentophilic fibrils, forming in several places a frame around the individual cells (Fig 6) Plasma cells might also be surrounded by reticular fibrils (Fig 10) There seemed to be a gradual transition from the argentophilic fibrils to coarser collagen-fibrils In the lymphocytic infiltrations, there was only sparse reticulin Sudan-stained frozen sections showed

primary localization in the submaxillary region does not accord with what Oberling has reported as characteristic of xanthogranuloma

The lesion in the submaxillary gland developed in connection with an acute illness, diagnosed as epidemic parotitis, but this is no proof of an inflammatory lesion Nor does the histological appearance permit aetiological conclusions, since cells derived from the histiocytic and reticular tissue may be encountered in inflammations, as well as in tumours Whether the disease may have been due to a virus infection or whether it is a neoplastic, reticulo histiocytic affection still remains an open question We are affronted with the same questions as in Hodgkin's disease which varies histologically from granulomatous lesions suggesting inflammation to purely sarcomatous infiltrations

When in 1935 Oberling published the first 6 cases of retroperitoneal xanthogranuloma he believed that the histological substratum was the same as in Schuller Christian's disease In 1957 a case of retroperitoneal xanthogranuloma, associated with visceral eosinophilic granuloma, was reported by *Waller, Hellwig & Barbosa*, and the same authors also mention another 8 cases from the literature including three of Oberling's cases (Table 1) In their opinion both tumours were a manifestation of the same disease entity Although the xanthogranuloma has some features in common with Hand Schüller Christian's disease, it seems justified to consider it a disease *sui generis* with a certain range of variation in the histological picture In the present case there were furthermore a very great number of plasma cells and a high sedimentation rate

SUMMARY

A case of plasmocytic xanthogranuloma in a 64 year old woman is reported The disease, localized to the submaxillary gland, was treated surgically and radiologically Six years later the right kidney was removed due to a new localization of the same disease The histological changes are described and the relation of this disease to Oberling's retroperitoneal xanthogranuloma is discussed

REFERENCES

Ackermann L V Tumors of the Retroperitoneum Mesentery and Peritoneum Atlas of Tumor Pathology Sect VI Fasc 23 24 [illegible]

Berg [illegible]

Besk [illegible]

Dietr [illegible]

[illegible] na of Bone 1944

[illegible]

of the infiltrated renal tissue showed a marked increase of the cholesterol content (0.185 per cent wet weight, while the normal level is 0.103 per cent). The total lipid amounted to 6 per cent of the dry weight. The lipophosphate was not increased (less than 0.05 per cent). (Analysis carried out by *Borge Larsen*, M.Sc.)

The patient was discharged about 3 weeks after admission, 10 days after the operation, and she is still well.

After discharge the E.S.R. was found to be 111 mm. The total lipid in the blood was 840 mg per cent, cholesterol 165 mg per cent. Apart from a fairly marked halisteresis, X-rays of the skeletal system showed no abnormality, especially no focal lesions.

DISCUSSION

The present case is interpretable as focal xanthomatosis without elevation of the cholesterol in the blood. It differs so much, in site and histological appearance, from Hand-Schuller-Christian's disease that it can hardly be an atypical form of this disease. It is worth emphasizing particularly the extremely varied histological picture showing transitions from cellular areas with histiocytes and reticulum cells to connective tissue of varying density and in places of an almost cicatricial character. In Hand-Schuller-Christian's disease the tissue changes show a more gradual development.

Histologically, the case is more suggestive of retroperitoneal xanthogranuloma as described by *Oberling*. In his cases, too, there were histological changes in which the polymorphism is emphasized as striking. Oberling considered the disease distinctly inflammatory with transition to sarcomatous areas.

As already mentioned, the present case showed cellular areas as well as more acellular ones with ample connective tissue. In some places, the cellular areas consisted almost exclusively of histiocytes, while in most areas they showed a motley mixture of histiocytes, reticulocytes, lymphocytes, and plasma cells. Especially the latter were strongly predominant in many areas, being present partly in clusters of varying density and partly in the form of sheaths around immature vessels, mainly of capillary nature. In relation to the plasma cells as well as the reticulocytes there was ample reticulin, frequently framing the individual cells. The histiocytic cells would in many places contain sudanophilic granules. In other places there was extra cellular cholesterol in clusters in the tissue. In the connective tissue there were, apart from plasma cells and lymphocytes, clusters of foam cells, most ample in sections from the kidney tissue.

Although in the main the histological picture corresponds to that described by Oberling, it differs in the very marked predominance of plasma cells, which also showed much more variation in shape, size, and nuclear size than usual in inflammatory lesions. Furthermore, the

FROM THE HISTOLOGICAL DEPARTMENT, UNIVERSITY OF UPPSALA SWEDEN
(HEAD PROF M WRETE)

THE ELASTIC COAT OF THE ARTERIAL WALL STUDIED WITH THE AID OF COLLAGENASE

By

O HASSLER, M WIREN and S HERBERTSSON

Received 13 iv 62

Collagenase can be distinguished from other proteolytic enzymes by the fact that it digests native collagen at physiological temperatures and pH Other enzymes such as pepsin or trypsin can only attack denaturated collagen

Collagenase was first described as a component of the group of toxins liberated by various members of the anaerobic organisms which produce gas gangrene (*Macfarlene & Mac Lennan* 1945) The only active collagenase preparations which have been described and confirmed are those produced from culture filtrates of Clostridium histolyticum and Clostridium Welchii Under certain conditions, collagen can be rendered soluble by mammalian proteinases (*Grant & Alburn* 1960) These authors suggested that the rapid destruction of collagen which probably occurs in vivo is enzymatic Purified preparations of collagenase have been described by *Mandl, Zipper & Ferguson* (1958) and by *Seifter, Gallop, Klein & Meilman* (1959)

Collagenase is assayed according to the method of *Mandl, Mac Lennan & Howes* (1953) in which collagenase is incubated for 18 hours with native collagen The extent of collagen break-down is determined using the photometric ninhydrin method The amino acids which are liberated are expressed as milliequivalents of leucine per mg collag-

m

Ac ... *& ... 1959, Hamit & Upjohn* 1960) It has been used in the preparation of tissues for tissue culture (*Hinz & Syverton* 1959)

MATERIAL AND METHODS

The material consisting of segments 2 cm long from the thoracic aorta and the basilar artery of 10 individuals aged 0–73 years was obtained at autopsy within 12 hours post mortem

Each segment was divided into two pieces One piece was treated with collagenase while the other served as a control.

Letterer F Über eine xanthose Lymphogranulomatose mit besonderes Beteiligung des Skelettes Verh d Deutsch path Gesellsch 31 Tag 1938 p 6 (1939)
Lichtenstein L Histiocytosis A M A Arch of Path *56* 84 1953
Melicow M M Xanthic lesions report of 4 cases includ two of xanthomatosis of kidney J A M A *105* 768 774 1935
Melicow M M Primary tumors of retroperitoneum clinicopathologic analysis of 162 cases review of literature and tables of classification J Internat C II Surgeons *19* 401–449 1953
Noel R & Michel Bechet R Xanthome perirenal intratesticulaire droit et sarcome fuso cellulaire perirenal gauche Ann d anat path *10* 215 1933
Nethen Ein Fall von Fibroxanthosarcom Frankfurt Zeitschr f Path *23* 417 418 1920
Oberling J Retroperitoneal Xanthogranuloma Am J Cancer *23* 477 489 1935
Tannhauser S J Lipidoses Oxford University Press New York 1950
Waller J I Hellwig C A & Barbosa F Retroperitoneal Xanthogranuloma Associated with Visceral Eosinophilic Granuloma Cancer *10* 388–392 1957

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Collagenase was first described as a component of the group of toxins liberated by various members of the anaerobic organisms which produce gas gangrene (*Macfarlene & Mac Lennan* 1945) The only active collagenase preparations which have been described and confirmed are those produced from culture filtrate [illegible]
Clostridium Welchii Under certain [illegible]
dered soluble by mammalian proteina [illegible]
authors suggested that the rapid destruction of collagen which probably occurs in vivo is enzymatic Purified preparations of collagenase have been described by *Mandl, Zipper & Ferguson* (1958) and by *Seifter, Gallop, Klein & Meilman* (1959)

Collagenase is assayed according to the method of *Mandl, Mac Lennan & Howes* (1953) in which collagenase is incubated for 18 hours with native collagen The extent of collagen break down is determined using the photometric ninhydrin method The amino acids which are liberated are expressed as milliequivalents of leucine per mg [illegible]

me [illegible]
Ac [illegible] It has been used in the preparation of tissues for tissue culture (*Hinz & Syverton* 1959)

MATERIAL AND METHODS

The material consisting of segments 2 cm long from the thoracic aorta and the basilar artery of 10 individuals aged 0–73 years was obtained at autopsy within 12 hours post mortem

Each segment was divided into two pieces One piece was treated with collagenase while the other served as a control.

The collagenase digestion was performed as follows The arterial specimen was placed in 10 ml of a 0 067 M phosphate buffer solution (pH 7 4), containing 0 45 per cent sodium chloride To this were added 20 mg of collagenase (Worthington Biochem Corp, Freehold, New Yersey) and 1 0 mg of chloramphenicol The solution was incubated at 37° C for 18 hours, after which the residue was transferred to a 5 per cent formaldehyde solution The control was treated in the same way except that the enzyme was omitted After the fixation, some specimens were stained in toto in aldehyde fuchsin and examined under a stereomicroscope (see *Hassler* 1961) Some other fixed specimens were examined by the optical sectioning technique using a slit lamp (*Brolin & Hassler* 1958 *Hassler* 1961) After fixation, most specimens were embedded in paraffin and divided into sections varying in thickness from 5-200 microns The thick sections were stained with aldehyde-fuchsin, orcein or resorcin fuchsin

The thin sections were investigated by *Wirén* and *Herbertsson* who used the following techniques:

a silver staining ad modum Foot (*Romeis* 1948) combined with periodic acid-Schiff's staining (PAS).
b alcian blue staining combined with PAS
c Hale's colloidal iron staining as modified by *Mowry* (1958)
d Gomori's aldehyde fuchsin staining as modified by *Eslelund* (1957)
e Barrnett & Seligman's SH-staining as modified by *Bahr* (1957)
f toluidine blue at pH 3 and 4
g haematoxylin eosin
h azan

RESULTS

The collagenase preparation dissolved all of the collagenous connective tissue and, in addition, most of the cytoplasm of the smooth muscle cells, an effect which might be attributed to an impurity in the preparation, *e g* contamination by some proteinase or peptidase The elastic component of the arterial wall remained, however, as did solitary cell nuclei which lay unattached and which therefore were easily washed free from the long elastic membranes and fibrils

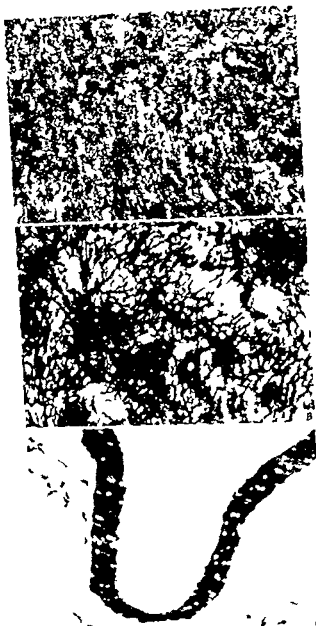
The coarse anatomical structure of the elastic coat could then easily be studied under the stereomicroscope (before embedding and sectioning) and on the thick paraffin sections after elastin staining (see Figs 1 *A-C*) No difficulties were encountered as compared with those found in the study where traditional maceration methods were used (*Brolin & Hassler* 1958)

A comparison, under high power magnification, between thin sections of the elastic tissue control and of that treated with collagenase, did not reveal any difference except in the sections investigated with the SH-staining technique In this instance the material treated with collagenase gave a positive stain (cf Fig 2) which was not specific, however, since it was also obtained after iodine oxidation

DISCUSSION

The elastic tissue forms one of the most important components of the arterial wall Pathological processes in the elastic tissue may play a considerable rôle in the development of arteriosclerosis and in the process of senescence

Fig 1
Arterial elastic tissue isolated with the aid of collagenase. A and B from human aorta. C the fenestrated internal elastic lamella of a human cerebral artery. (Aldehyde fuchsin and van Gieson. Magnification A × 35, B × 270, C × 240)



The microscopical study of the elastic tissue in the arterial wall is associated with some degree of uncertainty owing to the difficulty of obtaining an effective and completely selective agent for staining elastin (*Brolin & Hassler* 1961 and many others). All the elastin stains in common use have the disadvantage that they do not always stain all of th

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DISCUSSION

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elastic tissues Some elastin stains may produce a non selective staining of the collagenous connective tissue The resorcin-fuchsin agent frequently gives an unsatisfactory stain to the fine elastic fibrils of the arterial wall, although it always gives an excellent stain to the coarse elastic fibres of the human dermis (*Pearse* 1960, and others) The commercial orcein is a mixture of several components, some of which produce rather selective staining of the elastic tissue while others stain the collagen more strongly than the elastic tissue (*Brolin & Hassler* 1961, and others) Accordingly, a removal of the collagenous connective tissue may provide a basis for a more thorough study of the elastic component As is shown in this investigation, such a removal can be accomplished easily by means of a specific enzyme which digests the collagen

Previously, the elastic tissue component of the arterial wall has been isolated by maceration with alkali and acids However, these agents produce considerable changes in the staining properties and in the microscopical appearance of the elastic tissue (*Brolin & Hassler* 1958) After our collagenase treatment the only change that was found to occur was that a positive but non specific SH reaction was obtained It is doubtful whether this effect can be explained without further investigation It has been observed, however, that arterial elastic tissue from rabbits and rats fixed immediately after death also gives a positive reaction which is non-specific and probably due to an affinity for the naphtholic component of the reagent (*Barnett* 1953)

Hayward (1960) used collagenase for a similar application She found that collagenase was an excellent tool in the microdissection of kidney, and produced less alteration of the tissues than the traditional method of maceration with concentrated hydrochloric acid

SUMMARY

Isolation of the elastic tissue of the arterial wall is often desirable, for a number of reasons, and maceration with alkali or acids is usually applied for this purpose

In the present investigation, the enzyme collagenase was found to produce an effective isolation of the elastic component, which suffered less alteration than after treatment with acids or alkali The only change that could be found by histochemical examination was that a positive but non-specific SH reaction was obtained after digestion

REFERENCES

Bahr G F Changes in liver cell elements during stimulated protein synthesis A cytochemical study Acta Radiol (Stockh) Suppl *147* 1 1957

Barrnett R J The histochemical distribution of protein bound sulfhydryl groups J Nat Cancer Inst *13* 905 1953

Brolin S E & Hassler O Minute aneurysms detected by microdissection of basal cerebral arteries Acta path et microbiol scandinavica *44* 59 1958



Fig 2

Human cerebral artery *A* Not treated with collagenase *B* After collagenase treatment SH staining (Bahr's modification of Barrnett & Seligman's procedure) of the elastic lamella (arrowed) is negative in *A* but in *B* a positive (non specific) reaction is obtained Magnification *A* × 300 *B* × 450

FROM THE UNIVERSITY INSTITUTE OF HUMAN GENETICS COPENHAGEN (HEAD PROFESSOR TAGE KEMP, M.D.) DEPARTMENT OF EXPERIMENTAL GENETICS AND CYTOLOGY (J. SCHULTZ LARSEN, M.D.)—MUNICIPAL HOSPITAL, COPENHAGEN DEPARTMENT OF CLINICAL PATHOLOGY (HEAD SVEND PETRI, M.D.) —UNIVERSITY HOSPITAL OF COPENHAGEN, DEPARTMENT OF CLINICAL PEDIATRICS (HEAD PROFESSOR PREBEN PLUM M.D.)—THE CHILDREN'S HOSPITAL FUGLEBAKKEN, COPENHAGEN (HEAD FOLKE TUDVAD M.D.)

OVARIAN DYSGENESIS (TURNER'S SYNDROME) IN THE NEWBORN

Report of two Cases

By

ANDERS FRØLAND, ANNE LYKKE and BENGT ZACHAU-CHRISTIANSEN

Received 29 x 62

The aim of the present publication is to describe two cases of ovarian dysgenesis (Turner's syndrome) in newborn girls In both cases sex-chromatin and chromosome investigations have been carried out, in one in which the patient died three weeks old, an autopsy has been performed

CASE HISTORIES

1 CHF 686/61

The patient was readmitted 2 months later for further investigations A skin-biopsy from the back of the foot showed Oedema and moderate amounts of mucopolysaccharides extending down into the subcutis The sweatglands are embedded in fibrous tissue This and a thickening of the connective tissue in the epidermis point to a regeneration of connective tissue possibly because of oedema in organisation Skinbiopsy from the elbow showed normal features (sign G *Asboe Hansen*)

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Brolin S F & Hassler O Differences in staining elastic tissue between some orce n fractions separated on paper Acta Soc Med Upsal *66* 65 1961
Connell J F & Rousselot L M Use of enzymatic agents in debridement of burn and wound sloughs Surgery *30* 43 1951
Eskelund V Fast two step trichrome staining for paraffin and frozen sections combining demonstration of elastic fibres fibrin and mucus with v Gieson stain Acta path et microbiol scandinav *40* 105 1957
Grant N H & Alburn H E Collagen solubilization by mammalian proteinases Arch Biochem Biophys *89* 262 1960
Hamit H F & Upjohn H L Debridement of experimental burns with clostridium histolyticum H 4 enzyme Ann Surg *151* 589 1960
Hassler O Morphological studies on the large cerebral arteries with reference to the aetiology of subarachnoid haemorrhage Acta neurol et psychiat scandinav Suppl *145* 1 1961
Hayward N J Collagenase maceration of kidney for microdissecti n Nature (London) *186* 403 1960
Hin R W & Syverton J T Mammalian cell cultures for stu ly of influenza virus I Preparation of monolayer cultures with collagenase Proc Soc Exp Biol Med *101* 19 1959
Hooper N W & Ivins J C Wound debridement Arch Surg *79* 701 1959
Howes E L Use of protelytic enzymes as debridement agents Surgery *42* 948 1957
Howes E L Mandl I Zaffuto S & Ackermann W The use of clostridium histolyticum enzymes in the treatment of experimental third degree burns Surg Gyn Obs *109* 177 1959
Macfarlane R G & Mac Lennan J D The toxaemia of gas gangrene Lancet 249 328 1945
Mandl I Mac Lennan J D & Howes E L Isolation and characterization of proteinase and collagenase from clostridium histolyticum J Clin Invest *32* 1323 1953
Mandl I Zipper H & Ferguson L T Collagenase its purification and properties Arch Biochem Biophys *74* 465 1958
Mowry R W Improved procedure for the staining of acidic polysaccarides by Muller s colloidal (hydrous) ferric oxide and its combination with the Feulgen and the periodic acid Schiff reactions Lab Invest *7* 566 1958
Pearse A G F Histochemistry theoretical and applied Churchill Ltd London 1960
Romeis B Mikroskopische Technik Oldenbourg Munchen 1948
Seifter S Gallop P M Klein L & Meilman E Studies on collagen II Properties of purified collagenase and its inhibitor J Biol Chem *234* 285 1959

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CASE HISTORIES

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A two days old girl was transferred to a paediatric hospital The mother was 21 years old at the time of birth of the patient the father was 33 years The patient is a second child the older sib is apparently normal Pregnancy and delivery without complications Birth weight 2850 gr length 49 cm

Clinically the patient appeared normal (Fig 1) except for marked lymphoedema (Fig 2) on the back of the feet and slight cruaral oedema The latter disappeared in a few days There was no cubitus valgus nor webbing of the neck Heart sounds normal normal pulsation in femoral arteries External genitals normal Because of intermittent pyuria an intravenous urography was performed The right urogram showed normal conditions whereas the left disclosed slight dilation and caudal rotation

The patient was readmitted 2 months later for further investigations A skin biopsy from the back of the foot showed Oedema and moderate amounts of muc polysaccharides extending down into the subcutis The sweatglands are embedded in fibrous tissue This and a thickening of the connective tissue in the epidermis point to a regeneration of connective tissue possibly because of oedema in organisation Skinbiopsy from the elbow showed normal features (sign G Asboe Hansen)

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This investigation was aided by grants from the *Danish Atomic Energy Commission* the *F L Smith & Co Jubilee Foundat*[illegible] *of 21 12 1956 for the* [illegible] *esearch and Treat* [illegible] *dation the P Carl*



Fig 1

Patient no 1 at the age of three months In spite of the chromosomal abnormality (45/XO) the patient appears clinically normal except for the marked lymphoedema of the back of the feet



Fig 2

Patient no 1 at the age of three months The picture shows the lymphoedema of the back of the feet



Fig 3

Patient no 2 one week old Note webbing of the neck and low set, malformed ears

2 UHG 755/61

A two days old girl was transferred to a paediatric ward for further investigations The mother was 27 years old at the time of birth of the patient the father 34 years The mother had previously had two abortions in the 6th and the 5th month of gestation When two months had passed of the last pregnancy the mother presented signs of imminent abortion and was treated with proluton (R) injections (a progesteron preparation) receiving a total amount of 2 25 grams A slight hirsutism developed

Delivery was normal 1 2 weeks before term birth weight 2900 gr length 49 cm

At birth the child showed the following characteristics Malformed low set ears webbing of the neck (Fig 3) normal heart sounds but no pulsation in the femoral arteries Slight hypertrophy of the clitoris Marked lymphoedema of the back of the feet

The patient rapidly deteriorated she had frequent attaques of cyanosis and signs of cardiac failure A cardiac catheterization was planned but the child died two days later 25 days old

Autopsy

Examination of the following organs presented normal findings pleura lungs, bronchi, oesophagus, stomach, duodenum, the small and large intestines rectum, liver, spleen, kidneys, and adrenal glands Autopsy of the brain was not performed

Heart and large vessels the size of the heart is 5 5 × 3 cm The heart presents normal conditions, with the exception of an open foramen ovale, manifest as a slitlike opening, the length of which is 6 cm

Aorta cutting the ascending aorta shows a circumference of about 22 mm Just distal to the site from which the large vessels branch off

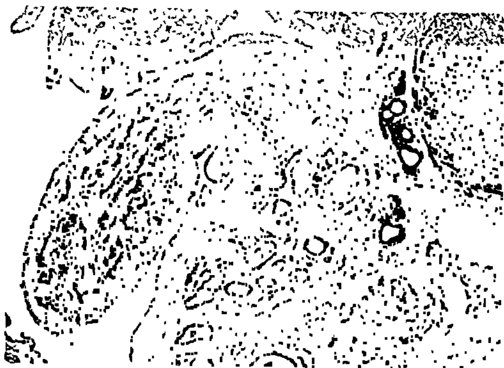


Fig 4
Low-power view of a section of the left gonadal region of patient no 2 The gonad is seen to the left, to the right a nodule composed of suprarenal cortical tissue

to the neck and upper extremities the aorta has narrowed The narrow part is about 1 cm long and the circumference is about 8 mm The ductus arteriosus is persistent, the diameter being about 1 5 cm It opens centrally into the narrowed area Distally to the narrowing the the circumference of the aorta is 18 mm

Genitals uterus, vagina and the tubes are of normal sizes and appearances according to the age of patient At the site of the ovaries two bean-shaped swellings are manifest, measuring about 5 × 3 cm

Histological Examination

Tissue specimens removed at the site of the gonads are cut serially On both sides cavities with a heavily convoluted mucosa are demonstrable Facing the lumen the tissue is lined by columnar ciliated epithelium characteristic of the uterine tubes Also a heavily convoluted system of ducts is demonstrable on both sides, the walls of which are composed of thick layers of concentric connective tissue cells and unstriated muscle cells Facing the lumen a cuboidal to low columnar, non-ciliated epithelium is seen arranged in a loose stroma of connective tissue, probably representing remnants of mesonephros Features characteristic of the seminiferous tubules or signs of spermatogenesis, are not demonstrated

A small pectiniform protrusion, a few mm wide, is demonstrable on



Fig 5
High power view of the left gonad of patient no 2 Note the very few primary follicles

the left side It is composed of ovarian stroma with filiform, closely-packed, fluctuating fibrils The surface is lined by a partly detached, low, cuboidal epithelium from which cell strands in many places lead to the stroma below The stroma is the site of a few primary follicles surrounded by a low, cuboidal epithelium (fig 4) No Graafian follicles are noted Some of the primary follicles contain a larger, eosinophil cell with remnants of nucleolus much reminding of the egg cell (fig 5) Elements reminding of Leydig cells or of "ovarian hilus cells" are not demonstrated

Close to the ovary is seen an equally large node, composed of cortical suprarenal tissue of normal appearance

Cytological Investigations

Sex-chromatin was determined on buccal smears stained by the Feulgen method Both patients were chromatin negative Chromosome-counts and analyses were performed on blood and/or skinbiopsies The method used for cultivating white cells from peripheral blood was the one described by *Moorhead et al* (1960) with slight modifications

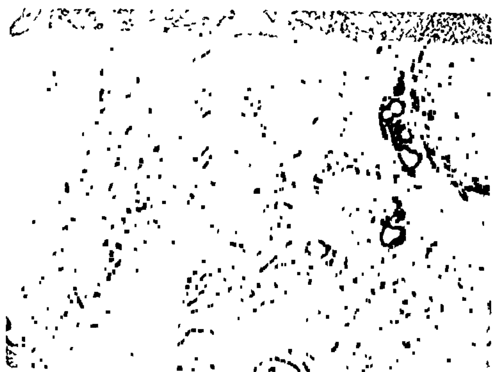


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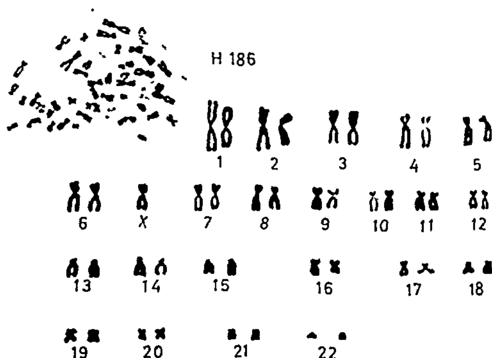


Fig 6

The chromosomes of patient no 2 Only 45 chromosomes and presumably only one X-chromosome Skin-culture

Fibroblasts were cultured from skin biopsies and prepared for chromosome investigations by the method of *Froland* (1961)

Case 1 Both blood cells and fibroblasts were studied From both tissues 30 cells were counted The distribution of the counts is seen in the table

TABLE 1

Distribution of Chromosome Counts in Case 1

Number of chromosomes	43	44	45	46	47	Total
Blood		2	28			30
Skin	1	3	25	1		30
Total	1	5	53	1		60

An analysis of 4 cells from each tissue with 45 chromosome showed only 15 chromosomes in group 6 12, incl X, whereas the other groups seemed to be those of a normal female As the patient is chromatin-negative it is probable that the caryotype is 45 (XO)

Case 2 At the time of the cytological investigations the patient was seriously ill and it was not thought safe to make a venipuncture nor a

bone marrow aspiration A skin biopsy was performed, and 30 cells grown in tissue culture were counted They all contained 45 chromosomes Here again it seems likely that the caryotype is 45 (XO) Fig 6

DISCUSSION

The term Turner's syndrome was originally applied to a syndrome in the grown up female (*Turner* 1938), symptoms including short stature, webbing of the neck, cubitus valgus and primary amenorrhoea Further it has been found that many of these patients suffer from malformations of various viscera Among the more important are the cardiac especially coarctation of the aorta, which often proves fatal Malformations of the kidney and ureters are common, but mostly asymptomatic Abnormalities of the genitals are of course more prominent in adults, in whom both internal and external genitals remain infantile In children the uterus, tubae, and vagina are mostly normal, whereas the clitoris may be enlarged (*del Castillo et al* 1957, *Gordan et al* 1955, *Ehrenfeld & Bromberg* 1958)

The most striking abnormality is seen in gonads These are long whitish structures replacing the normal ovaries

Microscopically they consist of dense fibrous tissue like that of normal ovarian stroma with filiform nuclei and wavy fibrills Only occasionally primordial follicles are seen (*Atria et al* 1948, *del Castillo & Argonz* 1957, *Greenblatt* 1958)

Cells resembling those of the testis have been described by *Grumbach et al* (1955), *Epps et al* (1958) and *Dougherty & Thompson* (1960), who found some cells of the Leydig type Others (*Guinet et al* 1954,*Gordan et al* 1955, *Nelson & Bailey* 1956) have described "ovarian hilus cells", which are presumed to have an androgenic function

Remnants of the mesonephros are not uncommon, nor of adrenal cortical tissue (*Dougherty & Thompson* 1960)

Most descriptions of post mortem findings in ovarian dysgenesis are based on observations in adults or older children (*Greenblatt* 1958) *Potter* (1961) found in a new born girl with symptoms of Turner's syndrome long narrow structures at the site of the ovaries Microscopically very fe[illegible]

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ratur[illegible] Turner's syndrome, ovarian or gonadal dysgenesis are the most common, whereas in continental literature the expression Bonnevie-Ullrich's syndrome often is encountered

Ullrich (1930, 1937, 1949) collected and described several cases and pointed out the possible relations between the lymphoedema of the feet the webbing of the neck, and the findings of *Bonnevie* (1934) in the *my* mice He also showed that the clinical signs may vary considerably

Since 1954 on several workers have shown that many patients with Turner's syndrome or ovarian dysgenesis were chromatin-negative like normal males (*Decourt et al* 1954, *Polani et al* 1954, *Wilkins et al* 1954) This led to the concept that a chromosomal abnormality might be the cause of the syndrome, but quite erroneously these patients were considered to have the sex-chromosomes of the normal male, XY This idea was based on the experiments of *Jost* (1947), who showed that early castration of the male foetus of the rabbit led to a female fenotype

In 1959 several workers (*Ford et al* 1959, *Fraccaro et al* 1959, *Jacobs & Keay* 1959, *Tjio et al* 1959) on the basis of observations of mitotic cells in tissue culture were able to conclude that the sex-chromosomes of these patients is neither of the normal female nor of the normal male types The patients have only one sex-chromosome, an X, and a total chromosome-number of 45, instead of the normal 46

Later it has been shown that numerous variations of the chromosomal abnormalities in ovarian dysgenesis exist The most important of these are. chromosomal mosaicism (*Ford* 1960, *Jacobs et al* 1960, 1961), X-isochromosome-formation (*Fraccaro et al* 1960), and deletion of the X-chromosome to a varying degree (*Jacobs et al* 1960, *de la Chapelle* 1962a)

Chromosomal investigations on a series of patients with Turner's syndrome have been carried out especially by *Jacobs et al* (1960,1961) and by *de la Chapelle* (1962b)

It was hoped that the determination of the chromosome complement in these patients would have made it possible to establish a clinical and cytological entity, but this has not as yet been the case

Our case 1 presented only one "classical" feature of the syndrome of Turner, namely the lymphoedema of the back of the feet Sex-chromatin and chromosome determinations, however, seem to establish the diagnosis The gonads are presumably dysgenetic ovaries

Patient 2 exhibited a variety of signs of ovarian dysgenesis lymphoedema, webbing of the neck, malformed ears, heart disease, and typical findings in the gonads The sex-chromatin and chromosome pattern were in accordance with the diagnosis

Whether the enlargement of the clitoris in this patient is part of the syndrome or it is due to the progesteron-treatment of the mother during pregnancy (*Wilkins* 1960) cannot be decided as the child died shortly after birth If the enlargement had been caused by the hormone-treatment, it would probably have regressed

As far as it is known to the authors a description of clinical, pathological and cytological findings in a newborn child with ovarian dysgenesis has not previously been published

SUMMARY

A report is given on two cases of ovarian dysgenesis (Turner's syndrome) in newborn girls The first had marked lymphoedema of the back of the feet but no other stigmata of the syndrome, the second exhibited a number of symptoms viz lymphoedema webbing of the neck, fatal coarctation of the aorta and at autopsy, streak gonads with abundant connective tissue with a few primordial follicles Both patients were sex chromatin negative and their chromosome pattern 45 (XO)

REFERENCES

Atria A Sanz R & Donoso S Necropsy Study of Case of Turner's Syndrome J Clin Endocrinol 8 397 1948

Bonnevie K Embryological Analysis of Gene Manifestation in Little and Bagg's Abnormal Mouse Tribe J Exp Zool 67 443 1934

del Castillo E B & Argon J Syndrome of Rudimentary Gonad Acta Endocrinol 62 379 1957

de la Chapelle A Chromosomal Mosaicism X Chromosome Anomaly and Sex Chromatin Discrepancy in a Case of Ganadal Dysgenesis Acta Endocrinol 39 175 1962 a

de la Chapelle A Cytogenetical and Clinical Observations in Female Gonadal Dysgenesis Acta Endocrinol Suppl 65 1962 b

Decourt [illegible] co

Doug [illegible] in

Ehrenfeld E N & Bromberg Y M Syndrome of Gonadal Dysgenesis with Enlarged Clitoris in Chromosomal Males Acta Endocrinol 28 540 1958

Epps D R Guerios M F M Neto A D S C Leon N Assis L M D & Cintra A B D U Clinical and Microscopical Findings in Gonadal Dysgenesis (Turner's Syndrome) 18 892 1958

Ford C E Jones K W Polani P E de Almeida J C & Briggs J H A Sex Chromosome Anomaly in a Case of Gonadal Dysgenesis Lancet i 711 1959

Fraccaro M Ikkos D Lindsten J Luft R & Kaiser K A New Type of Chromosomal Abnormality in Gonadal Dysgenesis Lancet ii 1144 1960

Fraccaro M [illegible]

Frola [illegible]

Gorlin G S Overstreet E W, Traut H F & Winch C A A Syndrome of Gonadal Dysgenesis A Variety of Ovarian Agenesis with Ande[illegible]

[illegible] 499 1954

Jacobs P A & Keay A J Somatic Chromosomes in a Child with Bonnevie Ullrich Syndrome Lancet ii 732 1959

Jacobs P A Harnden D G Court Brown W M Goldstein J Close H G MacGregor T N & Maclean N Abnormalities Involving the X Chromosome in Women Lancet i 1213 1960

Jacobs P A Harnden D G Buckton K E Court Brown W M King M J McBride J A MacGregor T N & Maclean N Cytogenetic Studies in Primary Amenorrhoea Lancet i 1183 1961

Jost A Sur les effets de la castration précoce de l'embryon male de lapin C R de la Soc de Biol., 141 126 1947

Since 1954 on several workers have shown that many patients with Turner's syndrome or ovarian dysgenesis were chromatin-negative like normal males (*Decourt et al* 1954, *Polani et al* 1954, *Wilkins et al* 1954) This led to the concept that a chromosomal abnormality might be the cause of the syndrome, but quite erroneously these patients were considered to have the sex-chromosomes of the normal male, XY This idea was based on the experiments of *Jost* (1947), who showed that early castration of the male foetus of the rabbit led to a female fenotype

In 1959 several workers (*Ford et al* 1959, *Fraccaro et al* 1959, *Jacobs & Keay* 1959, *Tjio et al* 1959) on the basis of observations of mitotic cells in tissue culture were able to conclude that the sex-chromosomes of these patients is neither of the normal female nor of the normal male types The patients have only one sex-chromosome, an X, and a total chromosome-number of 45, instead of the normal 46

Later it has been shown that numerous variations of the chromosomal abnormalities in ovarian dysgenesis exist The most important of these are chromosomal mosaicism (*Ford* 1960, *Jacobs et al* 1960, 1961), X-isochromosome formation (*Fraccaro et al* 1960), and deletion of the X-chromosome to a varying degree (*Jacobs et al* 1960, *de la Chapelle* 1962a)

Chromosomal investigations on a series of patients with Turner's syndrome have been carried out especially by *Jacobs et al* (1960,1961) and by *de la Chapelle* (1962b)

It was hoped that the determination of the chromosome complement in these patients would have made it possible to establish a clinical and cytological entity, but this has not as yet been the case

Our case 1 presented only one "classical" feature of the syndrome of Turner, namely the lymphoedema of the back of the feet Sex-chromatin and chromosome determinations however, seem to establish the diagnosis The gonads are presumably dysgenetic ovaries

Patient 2 exhibited a variety of signs of ovarian dysgenesis lymphoedema, webbing of the neck, malformed ears, heart disease, and typical findings in the gonads The sex-chromatin and chromosome pattern were in accordance with the diagnosis

Whether the enlargement of the clitoris in this patient is part of the syndrome or it is due to the progesteron-treatment of the mother during pregnancy (*Wilkins* 1960) cannot be decided as the child died shortly after birth If the enlargement had been caused by the hormone treatment, it would probably have regressed

As far as it is known to the authors a description of clinical, pathological, and cytological findings in a newborn child with ovarian dysgenesis has not previously been published

SUMMARY

A report is given on two cases of ovarian dysgenesis (Turner's syndrome) in newborn girls. The first had marked lymphoedema of the back of the feet, but no other stigmata of the syndrome, the second exhibited a number of symptoms, viz. lymphoedema, webbing of the neck, fatal coarctation of the aorta and at autopsy, streak gonads with abundant connective tissue with a few primordial follicles. Both patients were sex chromatin negative and their chromosome pattern 45 (XO)

REFERENCES

Atria A. Sanz R. & Donoso S. Necropsy Study of Case of Turner's Syndrome. J Clin Endocrinol., 8 397 1948
Bonnevie K. Embryological Analysis of Gene Manifestation in Little and Bagg's Abnormal Mouse Tribe. J Exp Zool. 67 443 1934
del Castillo E B & Argon J. Syndrome of Rudimentary Gonad. Acta Endocrinol., 62 379 1957
de la Chapelle A. Chromosomal Mosaicism. X Chromosome Anomaly and Sex Chromatin Discrepancy in a Case of Gonadal Dysgenesis. Acta Endocrinol. 39 175 1962 a
de la Chapelle A. Cytogenetical and Clinical Observations in Female Gonadal Dysgenesis. Acta Endocrinol. Suppl. 65 1962 b
Decourt L. da S Sasso W. Chiorbelli E. & Fernandes J M. Sobre o sexo genetico das pa entes ...

Ford C E. Jones K W. Polani P F. de Almeida J C & Briggs J H. A Sex Chromosome Anomaly in a Case of Gonadal Dysgenesis. Lancet i 711 1959
Fraccaro M. ... Type of Chromo... 1960
Fraccar... ... in Gonadal Dys...ge... 1959
Freland A. A Simplified Method for Making Chromosome Preparations from Skin Biopsies. Acta Path. et Microbiol. Scand. 53 319 1961
Gordan G S. Overstreet E W. Traut H F. & Winch G A. A Syndrome of Gonadal Dysgenesis. A Variety of Ovarian Agenesis with Androgenic Manifestations. J Clin E...

... Endocrinol. 15 499 1954
Jacobs P A. & Keay A J. Somatic Chromosomes in a Child with Bonnevie-Ullrich Syndrome. Lancet ii 732 1959
Jacobs P A. Harnden D G. Court Brown W M. Goldstein J. Close H G. MacGregor T N. ... ... e in Wo...
Jacobs ... *McBride* ... Amenorr...
Jost A. ... C R de

Moorhead P S Nowell P C Mellman W J Battips D M & Hungerford D A Chromosome Preparations of Leucocytes Cultures from Human Periferal Blood Exper Cell Res 20 613 1960
Nelson B M & Bailey B L Gonadal Dysgenesis and Associated Anomalies (Turner s Syndrome) A M A Arch Path 62 41 1956
Polani P E Hunter W F & Lennox B Chromosomal Sex in Turner s Syndrome with Coarctation of the Aorta Lancet ii 120 1954
Potter E L Pathology of the Fetus and the Infant Year Book Medical Publishers Inc Chicago 1961 p 470
Tjio J H Puck T T & Robinson A The Somatic Chromosomal Constitution of Some Human Subjects with Genetic Defects Proc Nat Acad Sci 45 1008 1959
Turner H H A Syndrome of Infantilism Congenital Webbed Neck and Cubitus Valgus Endocrinology 23 566 1938
Ullrich O Über typische Kombinationsbilder multipler Abartungen Ztschr Kinderh 49 271 1930
Ullrich O Zur Phänogenese kombinierter Missbildungen Montsschr Kinderh 68 94 1937
Ullrich O Turner s Syndrome and Status Bonnevie Ullrich Am J Human Genet 1 179 1949
Wilkins L Masculinization of Female Fetus due to use of Orally Given Progestins J A M A 172 1028 1960
Wilkins L Grumbach M M & van Wyk J J Chromosomal Sex in Ovarian Agenesis J Clin Endocrinol 14 1270 1954

FROM THE UNIVERSITY DEPARTMENT OF PATHOLOGY GENERAL HOSPITAL MALMÖ SWEDEN

STUDIES OF VASCULAR CHANGES IN CHRONIC LUNG CONGESTION

2 *Lymphatic Vessels*

By

GIUSEPPE FICHERA[1] and INGA HÄGERSTRAND

Received 28 vi 62

Though the lymphatic pathways in the lungs have long been known (*Rudbeck* 1653 *Willis* 1675) (17, 19) and though changes in these lymphatics may impair lung function, their pathology has received only little attention This also applies to lymphatic changes in chronic lung congestion, in which changes in the blood vessels have been the subject of extensive investigations Recently, however, a few authors have also focused interest on the pathology of the lymphatics Thus *D Arrigo* (3 4) (1959, 1960) studied the lungs in mitral stenosis and *Barroso Moguel* (1) (1959), lungs with secondary chronic hypertension of varying aetiology Both describe a mural thickening in the form of fibrohyalinosis of all the lymphatic branches from the interlobular vessels to the smallest lymphatic satellites of the arterioles and venules *Heath* (10) (1960) observed dilatation of the lymphatic vessels in chronic pulmonary hypertension secondary to mitral valve disease The significance of these changes and the role they play in the aetiology and pathogenesis of fibrosis in pulmonary congestion prompted us to study a fairly large series of chronically congested lungs in detail

MATERIAL AND METHOD

The material obtained at necr Co

... Weigert's e the lymphatic vessels

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RESULTS

The appearance of the lymphatics in our cases of chronic lung congestion secondary to mitral valve disease was essentially the same as in the cases of lung congestion secondary to other diseases The following description therefore applies to both groups

The main changes consisted of a fibrohyalinotic thickening of varying degree of the walls of the lymph vessels Such changes were found in 70 per cent of our series, in 15 per cent the fibrohyalinosis was fairly advanced This fibrohyalinosis could be followed from the intrapleural and interlobular branches (Figs 1–4) to the deep lymphatics, peribronchial, periarterial and perivenous (Figs 5–9) and also in walls of lymphatic satellites of small blood vessels, arterioles, venules and capillaries, even from alveolar ducts (Figs 10–12) The fibrohyalinotic thickening involved the walls of the lymphatics along the surface nearest the blood vessels as well as the aspect facing the respiratory cavity and sometimes it appeared as if the walls of the lymphatic vessels were predominant in the vascular connective tissue septa

The lymphatics were tortuous, sometimes with plexiform formations, and sometimes they ran spirally around the blood vessels In many cases (about 20 per cent) the lymphatics were more or less markedly distended It should be mentioned that when the lymphatics are dilated, their calibres are as large as or larger than those of the blood vessels they accompany It was sometimes difficult or impossible to recognize the lymphatics Topographically, however, their course could be traced thanks to a peculiar hue in sections stained according to van Gieson, in which the lymphatics showed up clearly against the connective tissue in the walls of neighbouring vessels and bronchi This bright, red hue often differed from the colour of the collagenous structures of the walls of the blood vessels, even when the latter were sclerotic

The lumina of the lymphatic vessels, when they could be recognized, were often empty and only seldom contained coagulated fluid or erythrocytes The endothelial lining of the walls of the lymphatics could be distinguished right out into the finest branches though sometimes with difficulty Valvular formations were seen not only in the

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Figs 1 4

Fig 1 Fibrous thickening of lymphatics in visceral pleura From a 37 year old man with chronic lung congestion for 6 years because of cardiac infarction Van Gieson × 380

Fig 2 Dilated lymphatic vessels in visceral pleura From a 33 year old woman in the control series with carcinoma of the uterine cervix and metastatic growth in the small pelvis Van Gieson × 380

Fig 3 Interlobular lymphatic with fibrous thickening of the wall From a 70 year old woman with a long history of cardiac incompensation because of hyper tension and coronary sclerosis Van Gieson × 120

Fig 4 Fibrous thickening of interlobular lymphatic vessel in higher magnification (same as in Fig 3) Van Gieson Weigert s elastica × 480



Figs 1–4



F gs 5 8

superficial lymphatics of the lungs (subpleural and interlobular), but also in the deep (peribronchial and perivascular) Elastic fibres were sometimes observed in large lymphatics

A clear correlation was found between the fibrohyalinosis of the lymphatic vessels and the severity of the blood vessel changes (5) Only in a few cases was hyalinosis of the lymphatic vessel walls seen in the absence of marked involvement of the blood vessels, and even then it was only slight The most severe cases of fibrohyalinosis of the lymphatic vessels also showed advanced changes of the blood vessels

DISCUSSION

The investigation confirmed the occurrence of fibrohyalinotic thickening of the walls of the pulmonary lymphatics in chronic lung congestion, a finding first described in detail by *D Arrigo & Barroso-Moguel*

D Arrigo regarded lymphatic fibrosis as a primary change in chronic lung congestion In the present investigation changes occurred almost invariably in both blood vessels and lymphatic vessels and were usually of about the same degree of severity Thus the fibrosis of the lymphatics would appear to develop simultaneously with other pathological changes occurring in chronic congestion In contrast to what is seen in haemosiderosis however, no signs of regression of the lymphatic fibrosis are to be expected *Barroso Moguel* expressed the view that fibrosis of the lymphatic vessels is due to abundant amounts of fluid and other pathological material (disintegration products) It is known that protein-containing fluid stimulates the proliferation of connective tissue (17), and stagnation of lymph is perhaps the most plausible cause of the thickening of the walls of the lymphatics We found no correlation between the accumulations of blood pigment and fibrosis of lymphatic vessels

Like earlier workers in this field (*Harrison* (9), *Heath* (10), *Oglesby* (15), *Gough* (7), *Fleischner* (6), D'Arrigo (3, 4) and *Barroso Moguel*

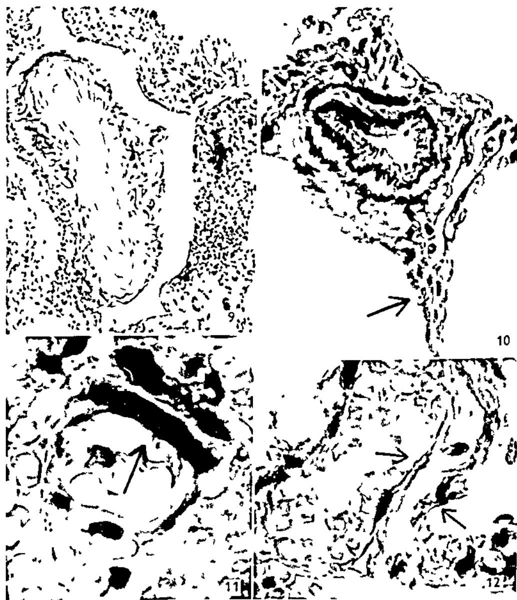
Figs 5–8

Fig 5 Wide periarterial lymphatics with valves From a newborn child in control series Van Gieson × 120

Fig 6 Lymphatic vessels with fibrous thickening near muscular artery From a 52 year old woman who had been operated upon 6 years previously with commissurotomy because of mitral stenosis Symptoms of cardiac failure recurred a few months after operation [illegible]

Fig 7 Periarterial lymphatic vessel From a 52 year old woman [illegible] sis with cardiac failure for 8

Fig 8 Fibr hyalinosis of periarterial lymph vessel (in upper part of artery) No distinct lumen is seen Observe the intensity of the stain *From a 38 year* old man with a 7 years *history* of cardiac failure because of mitral stenosis Van Gieson × 480



Figs 9–12

Fig 9 Periarterial dilated lymphatics with a focus of reticuloendothelial cells and carbon pigment outside vessel From a 59 year old man who died from haemorrhagic diathesis (because of antiprothrombin treatment?) Control case Van Gieson × 120

Fig 10 Arteriole in alveolar duct with muscular medial hyperplasia and intimal fibrosis Fibrous thickening of lymphatic vessel which can be traced out to the alveolar septa From same case as in Fig 7 Van Gieson Weigert's elastica × 380

Fig 11 Blood capillary with thickened lymphatic vessel Van Gieson × 900

Fig 12 Fibrohyalinosis of lymph vessels along blood capillary × 900

(1)), we often (20 per cent) found the lymphatics to be dilated But we cannot agree with *Heath* (10), who claims that the dilatation of the lymphatic vessels is the main change and that fibrosis does not occur Dilatation of the lymphatic vessels also seems to occur mainly in acute lung oedema (*Tobin* (21), *Harrison* (9)) We have observed this also in acute pulmonary oedema in some of our controls

It should be mentioned that in the beginning it was not so easy to detect the lymphatic vessels, particularly the small branches in the control material except in newborns, in whom it is easy to follow the course of the lymphatics because they are normally wide (Fig 5) No fibrohyalinosis could be demonstrated in the walls of the lymphatics in the control material but the very thin collagenous connective tissue fibres which are normally seen in the walls of the lymphatic vessels, were found to increase slightly with age

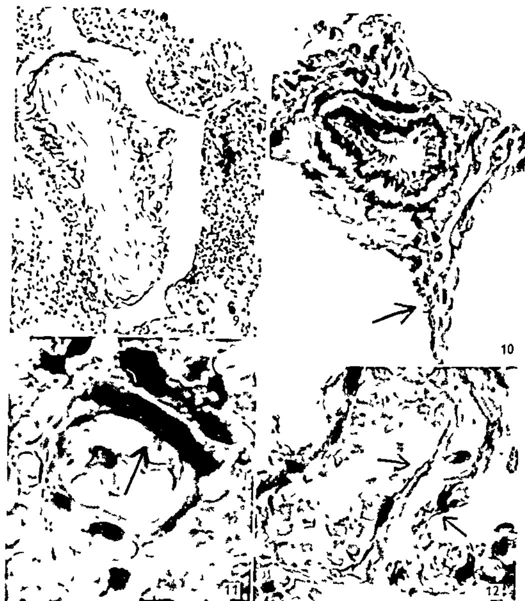
In accordance with the general conception, we were able to distinguish an endothelial lining right out to the smallest branches of the lymph vessels and we must therefore question the assertion of *Barroso-Moguel* that lymphatic vessels have no endothelial lining

Many authors have claimed (*Miller* (13, 14), *Maximow* (11)) that valves occur only in the superficial lymphatic pathways of the lungs, while others (*Heath* (10), *Barroso-Moguel* (1)) have described valves also in the deep lymphatic system of the lungs Our observations confirm the findings of the lastmentioned authors

Opinions differ on the distribution of the small lymphatic vessels *Renyi Vamos* (18) denies the occurrence of lymphatic vessels in alveolar septa except in alveoli immediately adjacent to the interlobar septa and adjacent to the large blood vessels *D Arrigo* claims that primary or secondary lymphatic vessels occur, though not regularly, in alveolar septa in chronic lung congestion secondary to mitral stenosis *Barroso-Moguel* (1) is of the opinion that lymphatic capillaries normally occur at least in the beginning of the alveolar septa They claim [illegible]

[illegible] lymphatics The present investigation will not allow any conclusion regarding the presence of lymphatics in alveolar septa or on the possible newfarmation of lymphatics in chronic congestion We have, however, been able to trace lymphatic capillaries in the proximal parts of alveolar septa thanks to the fibrosis of the walls of the lymphatics

In view of the number of lymphatics in the lungs [illegible]
we'' [illegible]
be [illegible]
ges [illegible] also be assumed to be a substantial function impairment owing to rigidity and lack of distensibility of the fibrotic lymphatic vessels As in other lung diseases, *e g* idiopathic pulmonary fibrosis *Meessen* (12), *von Hansemann* (8), *Vanek* (17))



Figs 9–12

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15 *Oglesby P Vawter G & coll* Patholog cal changes in congestive heart failure Arch of Path 64 363 1957
16 *Pall nger B & Florey H* Proliferation of lymphatics in inflammation J Path Bact 45 157 1937
17 *Rengi Vamos F* Das innere Lymphgefass system der Organe Ungarische Ak der Wissenschaften Budapest 1960
18 *Reny Vamos F & Papp B* The lymph vascular system of the lungs Act Anat 40 100 1960
19 Rus nyak J Foldi M & coll Lymphat cs and Lymph circulation Pergamon press Oxford 1960
20 *Soul e P Ba llet J & coll* Le pouman des m traux essai de confrontation anatomo phys ologique Arch Mal coeur 46 393 1953
21 *Tob n C* Lymphatics of the pulmonary alveols Anat Rec *127* 611 1957

the importance of changes in the lymphatics should be borne in mind also in the evaluation of chronic lung congestion

SUMMARY

The lymphatic vessels were studied in a necropsy series of chronic lung congestion (100 cases of mitral disease, 75 of varying aetiology) In 70 per cent of the cases generalized fibrohyalinosis of the lymphatics with thickening of the walls was found In about 15 per cent this change was severe In about 20 per cent, the lymphatics were dilated often in association with acute lung oedema

The fibrosis of the lymphatics may be due to the stagnation of lymph No correlation could be demonstrated between haemosiderosis and lymphatic fibrosis Fibrosis of the lymphatics is probably one of the main causes of the general fibrosis in chronic lung congestion To the functional insufficiency of the lymphatic system in stasis of the pulmonary circulation is added a mechanical insufficiency by fibrosis of the lymphatic pathways

It is pointed out that the lymphatic vessels, even their very fine branches, are lined with endothelium Valves occur in the superficial as well as in the deep lymphatic system of the lungs Lymphatic pathways can be traced out into alveolar septa

REFERENCES

1 *Barroso Moguel R & Costero I* The pulmonary lymphatic vessels in patients with hypertension of the lesser circulation Gac Med Mex *89* 525 1959

2 *Casley Smith J & Florey A* The structures of normal small lymphatics Quart J Exp Physiol *46* 101 1961

3 *D Arrigo S* The lymphatic vessel in the lung of mitral patients Boll Soc Ital Biol Sper *35* 1536 1959

4 *D Arrigo S Cordaro S & coll* Fibrosis of the lung of mitral patients Cuore Circol *44* 121 1960

5 *Fichera G & Hagerstrand I* Studies of vascular changes in chronic lung congestion 1 Blood vessels Acta pathol et microbiol scandinav In press

6 *Fleischner F & Reiner L* Linear X ray shadows in acquired pulmonary haemosiderosis and congestion New England J Med *250* 900 1954

7 *Gough J* Correlation of radiological and pathological changes in some diseases of lung Lancet *1* 161 1955

8 *v Hansemann D* Die Lymphangitis reticularis der Lungen als selbstandige Erkrankung Virch Arch *220* 311 1915

9 *Harrison C* The pathology of pulmonary vessels in pulmonary hypertension Brit J Radiol *31* 217 1958

10 *Heath D & Hicken P* The relation between left atrical hypertension and lymphatic distension in lung biopsies Thorax *15* 54 1960

11 *Maximow A & Bloom W* A Textbook of Histology Saunders Philadelphia 1955

12 *Meessen H* *Über* Lungencirrhose Beitr pat Anat u allg Path *110* 1 1949

13 *Miller W* Das Lungenlappen seine Blut und Lymphgefässen Arch f Anat 197 1900

14 *Miller W* The lymphatics and the lymphflow in the human lung Am rev Tuberc 3 193 1919

and seem to fuse (*Schultze* 1865, *Hayem* 1879, *Bizzozero* 1882, *Schimmelbusch* 1885) This alteration of the aggregates is considered necessary for rendering the platelet plug impermeable to blood, and it is induced by thrombin (*Luscher* 1956) The term "viscous metamorphosis" (*Eberth & Schimmelbusch* 1886 a) has unfortunately been used to designate various or all stages in the platelet transformation, although in our institutes the term has particularly been associated with the fusion of the platelets In this study we prefer to dispose of the term in favour of more exact expressions

During the last few years there has been serious doubt whether the *fusion* really implies loss of platelet individuality Several authors (*Policard, Collet & Giltaire Ralyte* 1955, *Rinehart* 1955, *Pease* 1956, *Levene & Levene* 1957, *Poole & French* 1961, *Iseri & Benditt* 1961, *Parmeggiani* 1961) have prepared sections of intravascular platelet thrombi or 'fused platelet aggregates *in vitro* and examined them by electron microscopy Aggregates of discrete platelets with fine boundaries have been revealed *Kjærheim & Hovig* (1962) found the same picture of densely aggregated individual platelets also in haemostatic platelet plugs In histological slides of platelet thrombi, produced *in vitro* and trapped in rabbit lungs, *Hand & Chandler* (1962) found that the platelets remained discrete for a long time, even after being engulfed by monocytes

In the histological studies on the platelet plug the finer structure was not investigated in detail *Apitz* (1942) stated that individual platelets could not be discerned, *M B Zucker* (1947) maintained that the plug appeared as a homogenous eosinophilic mass, and *H D Zucker* (1949) mentioned that the great majority of the platelets were fused

The question whether *fibrin* precipitation is essential to the formation of an efficacious platelet plug has been the subject of much debate Strands of fibrin in connection with the plug were noticed by several authors (*Hayem* 1882, *Apitz* 1942, *H D Zucker* 1949), but at most they considered them as a strengthening device of secondary importance *M B Zucker* (1947) did not find any fibrin in the plugs, neither *in vivo* nor in histological slides of the wounds fixed after half an hour On the other hand, no one denies the significance of an intact coagulation to the normal permanent haemostasis *Hugues* (1953) commented on this and concluded that fibrin was not present shortly after the cessation of the bleeding, but it appeared at a later stage This was neither denied nor confirmed by *Kjærheim & Hovig* (1962) because they did not clearly state whether fibrin was present at the time of haemostasis Before the cessation of the bleeding, at 45 seconds after the cutting, no fibrin was observed, while after the haemostasis, at 5 minutes after the cutting, fibrin fibrils appeared on the surface, but not within the plug

Little attention has been paid to the *further development* of the platelet plug *Lubnitzky* (1885) made arterial wounds in rabbits and

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UNIVERSITETETS INSTITUTT FOR TROMBOSEFORSKNING, RIKSHOSPITALET, OSLO. HEAD: PAUL A. OWREN, M.D.*

THE PLATELET PLUG IN NORMAL PERSONS

I. *The Histological Appearance of the Plug 15 to 20 Minutes and 24 Hours after the Bleeding and Its Role in the Capillary Haemostasis*

By

LEIF JØRGENSEN and CHRISTIAN F. BORCHGREVINK

Received 29.viii.62

Bleeding from small vessels is arrested by the formation of *platelet plugs*. When a small vessel is cut, blood platelets adhere immediately to the injured vascular wall and, within less than one minute, a platelet plug covers the mouth of the sectioned vessel. At first, the blood often leaks out intermittently, forming channels through the plug. However, they are rapidly filled by fresh platelets and, within a few minutes, the plug becomes impermeable and the bleeding ceases (*Hayem* 1882, *Lubnitzky* 1885, *Eberth & Schimmelbusch* 1886 b, *Apitz* 1942, *M. B. Zucker* 1947, *Chen & Tsai* 1947, *Hugues* 1953, *Witte* 1960, *Berman & Fulton* 1961).

Only few *histological* studies on the platelet plug have appeared in the literature of this century (*Ribbert* 1915, *Muller* 1931, *Apitz* 1942, *M. B. Zucker* 1947, *Hugues* 1953). The only comprehensive studies of this type in man are those of *Apitz* (1943) and *H. D. Zucker* (1949). *Apitz* (1943) made Duke's test for bleeding time in patients 12–24 hours *before death* and examined the wounds after death. *H. D. Zucker* (1949) made puncture wounds in skin of anaesthetized surgical patients and excised the wounds 15–20 minutes afterwards. *Monto* (1961) gave a short demonstration of the histological picture of the human platelet plug, obtained by the same technique as *H. D. Zucker* (1949). Quite recently, *Kjaerheim & Hovig* (1962) introduced electron microscopy of ultra-thin sections for studying the morphology of the platelet plug.

At extravasation and also at damage to the non-sectioned vascular wall, morphological and functional alterations of the platelets are rapidly induced. They become swollen and their form changes from a round disc to a globular or star-shaped body with filamentous processes. Functionally, they become adhesive and aggregate. Both *in vivo* and *in vitro* the aggregates are first composed of well-defined platelets, but within a few minutes the platelets loose their outlines

and seem to fuse (*Schultze* 1865 *Hayem* 1879 *Bizzozero* 1882 *Schimmelbusch* 1885) This alteration of the aggregates is considered necessary for rendering the platelet plug impermeable to blood and it is induced by thrombin (*Luscher* 1956) The term viscous metamorphosis (*Eberth & Schimmelbusch* 1886 a) has unfortunately been used to designate various or all stages in the platelet transformation although in our institutes the term has particularly been associated with the fusion of the platelets In this study we prefer to dispose of the term in favour of more exact expressions

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Little attention has been paid to the *further development* of the platelet plug *Lubnitzky* (1885) made arterial wounds in rabbits and

prepared sections of them from 6 seconds to 5 days after the bleeding The structure of the plugs changed from coarsely to finely granular and, finally, it became nearly homogeneous, giving the histochemical reactions of fibrin *Aschoff* (1892) gave a similar description of the transformation of platelet aggregates in intravascular thrombi

There is no general agreement about the mechanism of *capillary haemostasis* *Tocantins* (1947) stressed the importance of the tissue pressure, causing compression and collaps of the capillaries *Herzog* (1925) concluded that the lips of the sectioned capillaries have a great tendency to stick together at the transection, thus preventing bleeding The same observation was also made by *Apitz* (1942) and *Chen & Tsai* (1947), and it has been commonly accepted that this mechanism is often in operation However, *Apitz* (1942) also meant to have found evidence that capillary bleeding from parenchymatous organs is checked by precipitation of a superficial crust covering the bleeding surface His theory of two types of haemostasis, one for the capillaries depending on coagulation, another for small arteries and veins, depending on formation of platelet plugs, has been adopted by several authors (*H D Zucker* 1949, *Roskam, Hugues, Bounameaux & Salmon* 1959, *Witte* 1960)

In this study we shall report in detail the histological appearance of the normal human platelet plug formed in a standard wound and examined 15–20 minutes and 24 hours after the bleeding The problem of platelet fusion and the relationship between the platelet plug and fibrin formation will be discussed We are also going to comment upon the question of capillary haemostasis

This paper concerns only platelet plugs formed during the *primary* haemostasis (*Borchgrevink & Waaler* 1958) Subsequent papers will discuss the picture of platelet plugs formed during the secondary haemostasis (*Jorgensen & Borchgrevink* 1963 a) and in patients with haemorrhagic diseases (*Jorgensen & Borchgrevink* 1963 b)

MATERIALS AND METHODS

Determination of the primary bleeding time was performed in five normal persons on two succeeding days All were men aged 24 to 36 years (Table 1) In all the bleeding time as well as the platelet count was within the normal range

The method used for determination of the bleeding time is a modification of the technique described by *Ivy Nelson & Bucher* (1941) developed by *Borchgrevink & Waaler* (1958) A blood pressure cuff is placed on the upper arm and inflated to 40 mm Hg On the volar side of the forearm a transverse cut 1 mm deep and 10 mm long *is made* with a new surgical blade Every 30 second the shed blood is carefully absorbed by a filter paper until the bleeding stops The normal range for the bleeding time with [illegible]

In four of [illegible] wound of the first day was provoked in [illegible] time By so d[illegible]ing some of the platelet [illegible] it as it turned out several remained *in situ*

Biopsies Fifteen to twenty minutes after the cessation of the bleeding from the wound of the second day both wounds together with the surrounding skin were excised under local anaesthesia with xylocain 1 per cent solution

TABLE 1

Age Bleeding Time and Platelet Count in the 5 Normal Men from whom the Biopsies Were Taken

Person	Age in years	Bleeding time in minutes	Platelet count per cu mm blood
M H	31	6	271 000
D H	29	5	243 000
H S	24	5½	264 000
C B	36	7	237 000
P H	36	6	295.000

Histological technique The biopsy specimens were immediately transferred to Helly s solution for fixation for 24 hours Afterwards the specimens were rinsed in flowing tap water for another 24 hours imbedded in paraffin and cut in serial sections about 5 microns thick perpendicular to the direction of the wound The following stains were used

t),
nt
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ed

brown the fibrin dark blue

RESULTS

Biopsies of the Wounds 15–20 Minutes after the Bleeding

There was no important difference between the five wounds, allowing a joint histological account Shaped like a V, the wounds extended down through the upper forth or third of the corium Several vessels had been cut, their diameters ranging from about 5 to about 70 microns Occasionally, arteries of the subpapillary net (*Spalteholz* 1927) had been transected Somewhat more frequently, divided capillaries just beneath the epidermis were encountered The majority of the divided vessels, however were capillary veins belonging to the two upper venous networks of the corium

Th

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sp shaped, or irregular, but their outline was always smoothly curved, unless a part of the plug was broken off, as was sometimes the case (Fig 1) Two or more plugs belonging to neighbouring

had the largest plugs, with diameters up to 20 or 25 times that of the vascular lumen The plugs of the veins and some of the capillaries had diameters ranging from 2 to 10 times that of the lumen The smallest capillary plugs were not or only slightly larger than the mouth of the vessel (Fig 6)

The plugs were composed of densely aggregated discrete platelets,



Fig 1 Large broken platelet plug belonging to the arteriole in lower left hand corner The multivesicular structure is not clearly revealed by this magnification The plug is encompassed by a dark ribbon the perimetric fibrin membrane Lendrum's stain × 300

Fig 2 Large platelet plug formed by fusion of two or three plugs belonging to vessels at the bottom of the picture The perimetric fibrin membrane is partly duplicated Traversing channels are filled by platelets and erythrocytes At right the bleeding is apparently still continuing Haematoxylin eosin orange fuchsin × 150



Fig 3 Capillary plug with large ballooned platelets and fibrin membrane near the surface within the plug Epidermis just outside the upper border of the picture *Lendrum's stain* × *300*

Fig 4 Capillary plugs and to the right part of a venous plug with traversing channel Epidermis at extreme left *Lendrum's stain* × *300*

their diameters varying from 1 to 10 microns Within the same plug the size of the platelets was fairly uniform The majority of them had taken a distinct balloon form, *i e* their interior appeared either to be empty or to be occupied by a poorly stained refractive material (Fig 7) This gave the plugs a characteristic multivesicular appearance In some of the platelets a single tiny particle was peripherally located The limit-

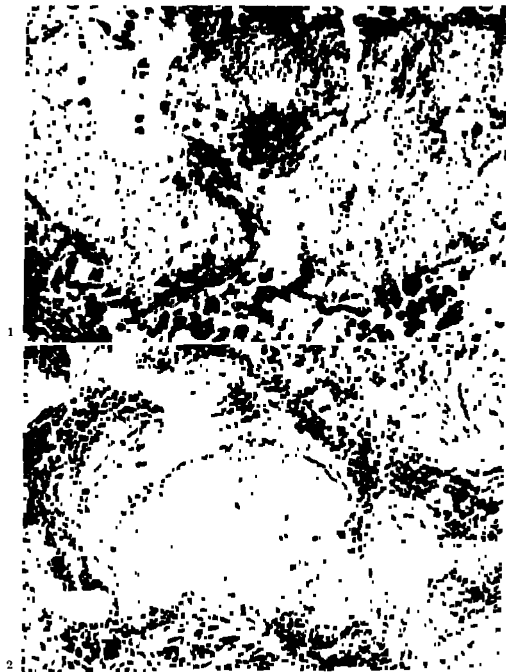


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ing platelet membranes appeared as curved fine strands which stained light blue to bluish red with Lendrum's stain and light brown with Mallory's phosphotungstic acid haematoxylin method At points where three of four platelets met the triangular or square spaces left were filled by cytoplasmatic masses of the same staining qualities Possibly, they represented platelet processes which had been trapped between densely packed platelets (*Kjærheim & Hovig* 1962)

Nearly the entire plug was lying outside the vessel within the gap of the wound, although occasionally platelet masses could be observed in the vessel at least as far as the diameter of the plug (Fig 5)

At the surface of the plugs, or near and parallel to it within the plugs, a membrane giving the staining qualities of fibrin was usually present Its thickness varied from about ½ to 4 microns In some of the larger plugs it was partly duplicated or triplicated It was made up of dense fibrin strands or it appeared as a differently coloured ribbon where the structure of the plug could be discerned right through The membrane was "secured' at the lips of the divided vessel and did not cross in front of its mouth Frequently, the perimetric membrane continued directly into the fibrin membrane covering the wound surface

A few plugs, particularly some of the capillary ones, lacked a perimetric membrane and were, in stead, diffusely stained as fibrin, in part or as a whole (Figs 5 and 6)

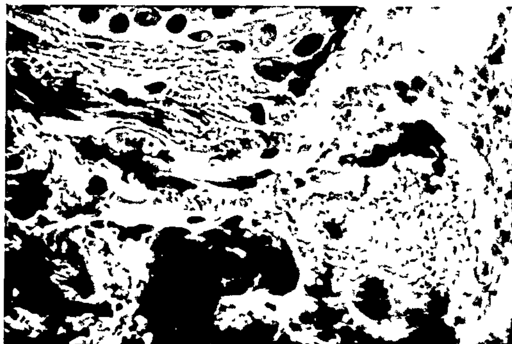
Through some of the larger plugs channels traversed in a straight or twisted course from the mouth of the vessel to the surface of the plug (Fig 2) The channels were mostly lined by a membrane identical and continuous with the perimetric membrane Often a few erythrocytes or leucocytes were lying within the channels, but most of the space was filled by ballooned platelets A few channels were completely filled by erythrocytes (Fig 2), probably indicating a continued minute bleeding at the excision in spite of the grossly apparent arrest

Some of the capillaries had not bled at all, their mouths being occluded by adhesion of the lips of the vessel (Fig 8)

The *walls of the wounds* were either naked or covered by a thin fibrin membrane, most constantly present near the skin surface (Fig 6 and 9) It was formed by a dense network of delicate strands to which tiny particles of platelet material were attached, often at the intersections of the fibrils

The *gap of the wound* was filled by erythrocytes, partly caught in a very fine fibrin net continuous with the membrane of the walls [illegible]
he- [illegible]
otl [illegible]

[illegible] the wounds were covered by a *superficial crust* composed of coarser fibrin net, haemolyzed erythrocytes and, in the outmost layer masses of aggregated platelets (Fig 10) The form of the platelets was round or serrated, their cytoplasm was granular, and many of them seemed to have fused Neither balloon forms nor any perimetric fibrin membrane were present here



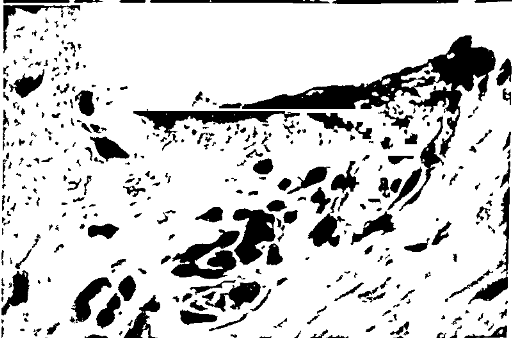


Fig 5 Uncommon capillary plug without perimetric fibrin membrane but with small areas of fibrin stain near the lower periphery of the plug. Aggregated platelets fill the vessel. Lendrum's stain × 400

Fig 6 Minute capillary plug staining diffusely as fibrin and blending with the fibrin membrane of the wall. Epidermis at upper left hand corner. Haematoxylin eosin orange fuchsin × 400



Fig. 11. Twenty four hours old platelet plug belonging to a dilated vein. The flattened granular plug has undergone complete fibrinous transformation. Lendrum's stain × 900.

Fig. 12. Twenty four hours old platelet plug belonging to a vein. The *perimetric* fibrin membrane is thickened, the platelets loosely aggregated and shrunken. Lendrum's stain × 300.

Biopsies of the Wounds 24 Hours after the Bleeding

Again the variations between the five wounds were small. The *platelet plugs* had diminished but their form was usually retained although some of the venous plugs had become flattened assuming a concavo-

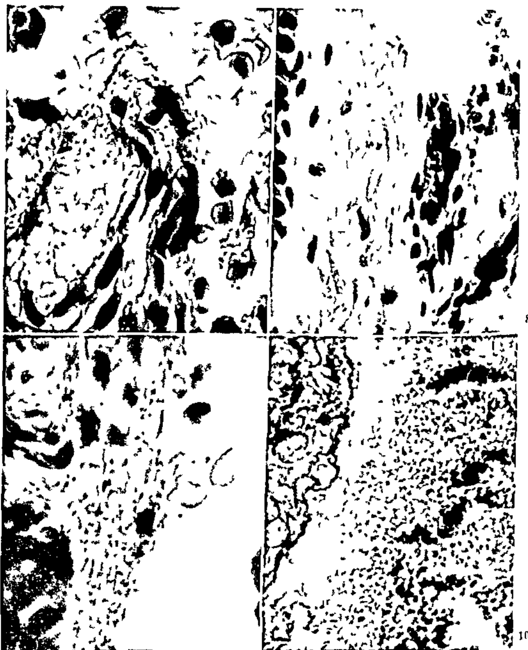


Fig 7 One of the capillary plugs in Fig 4 composed of densely packed swollen ballooned platelets some of which harbour a peripheral particle Lendrum's stain × 800

Fig 8 Capillary bleeding prevented by adhesion of the lips of the vessels Haematoxylin eosin orange fuchsin × 300

Fig 9 Fibrin net forming a superficial membrane on the wall of the wound Several tiny particles of platelet origin attached to the net Lendrum's stain × 900

Fig 10 Aggregate of apparently fused platelets in the outmost layer of the crust The fibrin net of the crust at left Lendrum's stain × 800

had apparently fused. Because the shed blood was absorbed every 30 second as long as the bleeding continued, these aggregates were probably fresher than the platelet plugs. We may surmise that the apparent fusion represents an early stage and the multivesicular structure a later stage in the transformation of the platelet aggregates. Support of this supposition is found in the literature. Both *Hayem* (1879) and *Schimmelbusch* (1885) recorded presence of vacuoles within the granular masses of fused platelets *in vitro* after a period of time. *Sharp* (1961), in a study on platelet aggregation and fusion in agitated plasma, watched the appearance of balloon-like structures in the granular masses at or just after the fibrin precipitation. These balloon forms contained actively motile black particles which tended to concentrate at the periphery. Our observations agree well with *Sharp's* description. In the electron microscope consistent changes were found by *Kjærheim & Hovig* (1962). After the haemostasis most of the organelles had disappeared and the density of the hyaloplasm was reduced in most of the platelets.

The apparent fusion is probably an artifact caused by the difficulty in distinguishing the cell borders when the granular platelets come very close to each other. The balloon forms could be the result of potocytosis, as proposed by *Sharp* (1961), but according to *Zollinger* (1948) this process is dependent on free cell surfaces in direct contact with excessive fluid. These conditions are not fulfilled in the platelet plug. Alternatively, *Sharp* thought it possible that these balloons might represent secretion of some unknown material. It is evident from our slides that the ballooning of the platelets is a reversible process, and that is compatible with a secretion which later empties.

We want to stress the fairly regular occurrence of a *perimetric fibrin membrane, present at least from 15–20 minutes after the bleeding*. Probably it is induced by the extravasated blood, actually on the contact surface between the blood and the plug. Another example of precipitation on a contact surface is the fibrin lining of the traversing channels, formed by leaking blood through the still permeable plug.

The fibrin network of the membrane of the walls, of the gap of the wound, and of the superficial crust had, 15–20 minutes after the bleeding, the same general structure as seen in histological sections of fibrin clots formed *in vitro* (*Benthaus & Grunberg* 1958, *Gaintner* 1959, *James, Johnson, Monto, Diab & Caldwell* 1962). The tiny particles were the only visible remnants of the platelets which had entered into the formation of the net. The contrast to the [illegible]
is obvious. In their electron- [illegible]
and *Parmeggiani* (1961) w[illegible]
between platelets in larger [illegible] within the small
aggregates for[illegible]
(*Braunstei*[illegible]
1962) and [illegible]

convex shape (Fig 11) In the middle and at the vascular pole of the largest plugs the platelets were not as densely packed as before (Fig 12) They had become shrunken and star shaped, the ballooning no more being conspicuous Many of the platelets had even a granular cytoplasm The fibrin membrane of these plugs was considerably thicker, and most of the middle-sized and smaller plugs had even undergone complete fibrinous transformation (Fig 11) Still the outlines of some of the shrunken platelets could be made out through the fibrinous material However, mostly the plugs had a diffusely granular structure, as if the platelets had fused within the fibrin mass Many small capillary plugs could be distinguished from the fibrin membrane of the walls only with difficulty

Both the membrane covering the *walls of the wounds* and the net within the *gap* were more constantly present and coarser than before, composed of thick, granular fibrin bands In the *surrounding tissue* an acute inflammatory reaction was evident

DISCUSSION

The *histological method* of studying the platelet plug gives only of a snapshot of a dynamic process Further, the observations made must be interpreted with due regard to possible artifacts These facts have probably discouraged most workers in the field of haemostasis from using this method However, at present it is the only practical way of studying the morphological aspects of the arrest of bleeding in man The detailed picture obtained lends itself to an interesting correlation with results of studies of haemostasis by other methods Moreover, the impossibility of observing the continuous progress of changes may in part be made up for by taking biopsies of a standard wound at intervals, and by noticing differences in degree of changes at the same point of time Finally, the histological picture of the normal platelet plug has a value of its own, irrespective of possible artifacts, particularly as a reference when studying pathological deviations (*Jorgensen & Borchgrevink* 1963 b)

In this study the normal platelet plug 15–20 minutes after the bleeding had a *structure* which departed from the conventional description of a granular mass of fused platelets In fact, the plug had a striking multivesicular appearance Reasonably, the vesicles must be interpreted as swollen or ballooned, densely aggregated discrete platelets Their limiting membranes were distinctly seen as curved fine strands, not taking the fibrin stain This morphological picture is in accordance with the electron microscopical studies on sections of the platelet plug and the intravascular platelet thrombus It confirms that the formation of an impermeable platelet plug does not imply loss of platelet individuality

On the other hand, many platelets of the aggregates within the crust

had apparently fused Because the shed blood was absorbed every 30 second as long as the bleeding continued, these aggregates were probably fresher than the platelet plugs We may surmise that the apparent fusion represents an early stage and the multivesicular structure a later stage in the transformation of the platelet aggregates Support of this supposition is found in the literature Both *Hayem* (1879) and *Schimmelbusch* (1885) recorded presence of vacuoles within the granular masses of fused platelets *in vitro* after a period of time *Sharp* (1961), in a study on platelet aggregation and fusion in agitated plasma, watched the appearance of balloon like structures in the granular masses at or just after the fibrin precipitation These balloon forms contained actively motile black particles which tended to concentrate at the periphery Our observations agree well with *Sharp*s description In the electron microscope consistent changes were found by *Kjærheim* & *Hovig* (1962) After the haemostasis most of the organelles had disappeared and the density of the hyaloplasm was reduced in most of the platelets

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The fibrin network of the membrane of the walls, of the gap of the wound, and of the superficial crust had 15–20 minutes after the bleeding the same general structure as seen in histological sections of fibrin clots formed *in vitro* (*Benthaus* & *Grunberg* 1958, *Gaintner* 1959, *James, Johnson Monto, Diab* & *Caldwell* 1962) The tiny particles were the only visible remnants of the platelets which had entered into the formation of the net The contrast to the ballooned platelets of the plugs is obvious In their electron microscopical studies *Iseri* & *Benditt* (1961) and *Parmeggiani* (1961) were also impressed by the great difference between platelets in larger aggregates and in clots [illegible]

1960) have shown loss of platelet individuality or integrity Consequently, there must be a difference in the platelet transformation according to which stimuli affect the platelets, in one case leading to the formation of a plug or platelet thrombus, in another to participation in the production of a fibrin net Unfortunately, the uncritical use of the term "viscous metamorphosis" has obscured this fact

As to the *further development* of the platelet plug, it is evident that the perimetric fibrin membrane becomes gradually thicker until the entire plug is a fibrin mass It is probably the platelets which lend a granular appearance to the transformed plug At the same time, the fibrin net becomes coarser with thick granular bands, and the striking structural differences between the plugs and the fibrin net are nearly obliterated

Some of the increase in the amount of fibrin both in the plugs and in the fibrin net may only be apparent, caused by retraction As first pointed out by *Benthaus & Grunberg* (1958), there is a marked enlargement of the platelet particles of the fibrin net during the retraction Further in an electron-microscopical study *Kuhnke* (1958) found considerable broadening of the fibrin strands during this process However, this alone can hardly account for the entire increase of fibrin It is not unlikely that a prolonged or continuous coagulation occurs in plasma leaking out into the wound, a leakage which may be advanced by the acute inflammatory reaction This would particularly explain the coarsening of the fibrin net Whether plasma also oozes slowly into the plug and clots, or whether clotting factors adsorbed onto the platelets (*Adelson, Rheingold & Crosby* 1961) are gradually activated in centripetal direction, precipitating the fibrinogen in or on the platelets (*Salmon & Bounameaux* 1958, *Schmid, Jackson & Conley* 1962), are open questions

According to our observations, the *capillary haemostasis* is effected by one of two means the bleeding is either arrested by the formation of a platelet plug, or the capillary has not bled at all, its mouth being occluded by endothelial adhesion The latter mechanism was not as commonly encountered as expected from the literature Probably, the proportion of capillaries occluded by endothelial adhesion to those that bleed varies with several factors, *e g* the species, the consistency of the tissue (*Apitz* 1942, *Roskam, Hugues, Bounameaux & Salmon* 1959), and the manner by which the wound is inflicted

The observation of capillary platelet plugs is at variance with previous authors using the histological technique Admittedly, *Apitz* (1942) did see capillary plugs in his *in vivo* experiments on rabbit mesentery, but only rarely However, he did not find these in the histological slides of experimental wounds neither in normal animals (*Apitz* 1942) nor in man (*Apitz* 1943) In the latter study the missing of the capillary plugs may be explained by the fact that he examined the wounds only 12 to 24 hours after the bleeding, at which time the capillary plugs had under-

gone complete fibrinous transformation and were blending with the fibrin membrane of the walls It remains unexplained why *H D Zucker* (1949) did not observe capillary plugs, because he took the biopsies of the wounds already 15–20 minutes after the bleeding time test

SUMMARY AND CONCLUSIONS

Determination of the bleeding time was performed in five normal persons and biopsies of the wounds were taken 15–20 minutes and 24 hours after the bleeding Platelet plugs covered the mouths of the divided vessels, even those of the majority of the capillaries The bleeding had been prevented from some of the capillaries by endothelial adhesion

Fifteen to twenty minutes after the bleeding the plugs were composed of densely aggregated ballooned platelets At the periphery of the plugs a fibrin membrane was usually present Within the wounds there was a network of thin fibrin strands with tiny particles of platelet origin Masses of apparently fused platelets were lying in the superficial crust

Twenty-four hours after the bleeding the plugs had become smaller and the platelets were shrunken, granular, and not as densely packed as before The perimetric fibrin membrane was thicker and most of the smaller plugs had undergone complete fibrinous transformation The fibrin net of the wound was coarse, composed of thick bands

The following conclusions are drawn

The formation of an impermeable plug does not imply loss of platelet individuality

The apparent fusion of platelets within larger aggregates, *e g* platelet plugs, is probably an artifact

The ballooning of the platelets *may be a further stage in the transformation of such aggregates It* may imply secretion of some unknown material, which at 24 hours has emptied

The fibrin membrane which encompasses the platelet plug 15–20 minutes after the *bleeding is* probably formed by contact with the extravasated blood

Because of the obvious *difference between the ballooned platelets* [illegible]

[illegible]

The plug are transformed *to fibrin* masses by gradual thickening of the perimetric membrane

The increase of *the fibrin in the* plugs and in the fibrin net 24 hours after the bleeding may *partly be* apparent, caused by retraction, partly real, caused by *prolonged or continuous coagula*[illegible]

[illegible]

Capillary bleeding is either arrested by platelet plug formation, or prevented by endothelial adhesion

REFERENCES

Adelson E Rheingold J J & Crosby W H The platelet as a sponge a review Blood *17* 767–774 1961

Apitz K Die Bedeutung der Gerinnung und Thrombose fur die Blutstillung Virchows Arch path Anat, *308* 540 614, 1942

Apitz K Die Dukesche Probe *Z* ges exp Med *111* 554 584 1943

Aschoff L Ueber den Aufbau der menschlichen Thromben und das Vorkommen von Plattchen in den blutbildenden Organen Virchows Arch path Anat *130* 93 144 1892

Benthaus J & Grunberg H Histologische Untersuchungen uber die Retraktion Thromb Diath haem *2* 140–145 1958

Berman H J & Fulton G P Platelets in the peripheral circulation Pp 7 20 in *Johnson S A Monto R W Rebuck J W & Horn R C Jr* eds Henry Ford Hospital International Symposium Blood Platelets London J & A Churchill Ltd 1961

Bizzozero J Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung Virchows Arch path Anat *90* 261 332 1882

Bloom G The morphology of human blood platelets and the coagulation of human blood in vitro An electron microscopical examination Z Zellforsch *42* 365 385 1955

Borchgrevink C F & Waaler B A The secondary bleeding time A new method for the differentiation of hemorrhagic diseases Acta med scandinav *162* 361–374 1958

Braunsteiner H Über Morphologie Physiologie und Pathologie der Thrombocyten Klin Wschr *29* 335–338 1951

Chen T I & Tsai C The mechanism of haemostasis in peripheral vessels J of Physiol *107* 280–288 1947

Eberth J C & Schimmelbusch C Experimentelle Untersuchungen uber Thrombose Virchows Arch path Anat *103* 39–87 1886 a

Eberth J C & Schimmelbusch C Experimentelle Untersuchungen uber Thrombose II Die Entstehung von Thromben in grosseren Gefassen von Saugethieren Virchows Arch path Anat *105* 331–350 1886 b

Gaintner J R Unpublished observations 1959 Cited by *Conley C L* Platelets in clot retraction Pp 437–444 in *Johnson S A Monto R W Rebuck J W & Horn R C Jr* eds Henry Ford Hospital International Symposium Blood Platelets London J & A Churchill Ltd 1961

Hand R A & Chandler A B Atherosclerotic metamorphosis of autologous pulmonary thromboemboli in the rabbit Am J Path *40* 469 486 1962

Hayem G Recherches sur l evolution des hematies dans le sang de l homme et des vertebrés Arch de Physiol *6* (ser 2) 201 261 1879

Hayem G Sur le mécanisme de l arret des hemorrhagies Compt rend Acad sci *95* 18 21 1882

Hertoj F Die Rolle der Capillaren bei der Blutstillung Pflugers Arch ges Physiol *207* 476 487 1925

Hugues J Contribution à l etude des facteurs vasculaires et sanguins dans l hémostase spontanee Arch Internat Physiol *61* 565 711 1953

Isert O A & Benditt F P Genesis of thrombi study of their fine structure Fed Proc *20* 133 1961

Ivy A C Nelson D & Bucher G The standardization of certain factors in the cutaneous venostasis bleeding time technique J Lab clin Med *26* 1812 1822 1941

James T N Johnson S A Monto R W Diab G & Caldwell J Histology of platelet plasma clots from normal subjects and patients with abnormal coagulation Blood *19* 751 763 1962

Jorgensen L & Borchgrevink C F The platelet plug in normal persons II The histological appearance of the plug in the secondary bleeding time test To be printed in Acta path et micr biol scandinav 1963 a

Jørgensen L & Borchgrevink C F The haemostatic mechanism in patients with haemorrhagic diseases A histological study of wounds made for primary and secondary bleeding time tests In preparation, 1963 b

Kjærheim A & Hovig T The ultrastructure of haemostatic blood platelet plugs in rabbit mesenterium Thromb Diath haem, 7 1–15, 1962

Kuhnke F Elektronenoptische Untersuchungen über die Veranderung der Thrombocyten und des Fibringerinnsels im Verlaufe der Gerinnung unter besonderer Berucksichtigung der Retraktion Pflugers Arch ges Physiol, 268 87 104, 1958

Lendrum, A C The staining of erythrocytes in tissue sections A new method and observations on some of the modified Mallory connective tissue stains J Path Bact, 61 443 448 1949

Levene M & Levene, C I Pulmonary platelet thrombo embolism J clin Path, 10 200–203 1957

Lubnitzky S Die Zusammensetzung des Thrombus in Arterienwunden in den ersten funf Tagen Arch exp Path Pharmakol, 19 185–208, 1885

Policard A Collet A & Giltaire Ralyte, L Étude au microscope électronique de thrombi plaquettaires formés dans les capillaires pulmonaires au cours d'agressions expérimentales par des particules inframicroscopiques de silice Bull Micr appl, 5 7–8 1955

Poole J C F & French J E

Ribbert H Die H[illegible]
bose Virchow

Rinehart J F He
telets With [illegible] the derivation of specific granules from mitochondria Am J Clin Path, 25 605–619, 1955

Rodman N F Jr

Roskc

ires et, en particulier,

Virchows Arch path

Schmid H J
telets J

Schultze M
des Blut [illegible] 42 1865

Setna S S & Rosenthal R L Intermediate stages in platelet alterations during coagulation Acta haemat 19 209 221, 1958

Sharp A A Platelet (viscous)
R W Rebuck J W & Hor

Etude morphologique et
ns Arscia S A., 1960

asse des Coriums insbesondere Papillarkreislauf Pp 400 421 in Jadassohn J ed Handbuch der Haut- und Geschlechtskrankheiten vol 1 1 Anatomie der Haut Berlin Julius Springer, 1927

Tocantins L M The mechanism of hemostasis Ann Surg *125* 292 310 1947
Witte S Morphologische Befunde uber die funktionellen Beziehungen zwischen Blutgerinnung und Blutgefassen Ingelheim am Rhein C H Boehringer S hn 1960
Zollinger H U Cytologic studies with the phase microscope I The formation of '*blisters in cells in suspension (potocytosis) with observations on the nature* of the cellular membrane Am J Path 24 545 568 1948
Zucker H D Platelet thrombosis in human hemostasis A histologic study of skin wounds in normal and purpuric individuals Blood 4 631 645 1949
Zucker, M B Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis in normal thrombocytopenic, heparinized and hypoprothrombinemic rats. Am J Physiol, *148* 275-288 1947

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DISAPPEARANCE RATE OF TRYPAN BLUE IN RAT PLASMA AFTER INTRAPERITONEAL INJECTION

By

HOLGER THILANDER

Received 13 xi 62

In order to ensure intense vital staining of the tissues repeated injections are recommended and intervals ranging from 2 to 5 days have been suggested (*Cappell* 1929 *Romeis* 1948) Since whatever the mode of administration the dye is transported from the site of injection to the various parts of the body via the circulatory apparatus, it is important to know the speed with which trypan blue disappears from the plasma after injection, for this will determine the minimum interval between the different injections

When using a method in which repeated intraperitoneal injections of trypan blue are given at fairly short intervals it is of interest to follow the variation in concentration of the dye in the plasma in the intervals between injections Any variation in this respect between different intervals would be attributable to a change in the resorption and excretion of the dye

The results of studies on this relationship after intravenous injection which have been performed by *Gregersen & Rawson* (1943) and [illegible]

[illegible] from the peritoneum

In order to examine this problem the following study was performed

MATERIAL AND METHOD

Two experiment [illegible]
were given one [illegible]
blue amounting [illegible]
taken from the [illegible] minutes and [illegible]
¾ 1½ 2¼ 3 3¾ 4½ 5¼ 6 7 9 12 15
for 11 days Each time about 40 [illegible]
[illegible]

with sample cells having a light path of 5 cm The zero was adjusted against distilled water On adding 10 µl of unstained plasma to 3 ml of distilled water no measurable absorption values could be observed

From the results of this experiment the optimum interval between intraperitoneal injections was taken as 24 hours In the second experiment one injection was given daily for 6 days in succession to each of 4 rats Following the first and last injections samples were taken during the next 24 hours according to the schedule of the previous experiment On each of the 4 intervening days one sample was taken immediately prior to the injection In other respects the method was as for the previous experiment

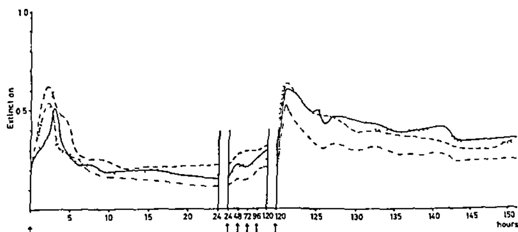


Fig 1

Relationship between time and extinction for plasma in 4 rats receiving one intraperitoneal injection of one per cent aqueous trypan blue on 6 days in succession The arrows mark the various times of injection

RESULTS

The results obtained during the first 24 hours of the second experiment, which are reproduced graphically in Fig 1, were similar to the corresponding results in the first In all of the animals the concentration of trypan blue in the plasma reached its maximum after about 2 hours The concentration fell sharply during the following two hours, and after 10 hours it had reached a value which thereafter diminished very slowly After 12 days there was still a detectable amount of trypan blue in the plasma

On repeated injections at 24-hour intervals the trypan blue in the plasma increased steadily Comparison of the extinction curves shows that the increase in concentration after the last injection was less than the corresponding increase after the first injection, and that after reaching its maximum value the concentration fell slowly and more steadily

The 4 animals suffered no ill effects from the administration of trypan blue and, at the end of the experiment, they were deep blue in colour

DISCUSSION

The resorption of dye from the sites of deposit evidently take place quite rapidly The curve for a single injection after the maximum con

centration had been reached is similar to the one after intravenous injection reported by *Gregersen & Rawson* (1943) and *Auskaps & Shaw* (1955), which, however, fell a little more rapidly The marked drop in the amount of trypan blue in the plasma during the first few hours after the maximum concentration has been reached has been ascribed to the type of bond between the dye and the plasma protein (*Rawson* 1943, *Gregersen & Rawson* 1943), whereby the trypan blue is linked primarily to the plasma albumin and only two molecules of the dye are linked to each albumin molecule Any excess of trypan blue will then be bound to the plasma globulins Since the capacity of the plasma protein to combine with trypan blue is limited, if the excess of dye is large part of it will remain free in the plasma and disappear rapidly from the circulation into the tissues and the urine It is this penetration into the tissues that accounts for the good staining properties of trypan blue

Because of the low toxicity of trypan blue and the fact that the plasma concentration falls slowly after 10 hours, the interval between injections could be reduced from the proposed 2-5 days to, say, 24 hours The continuous increase in plasma concentration in the 24-hour tests appears to be due to an increase in the amount of free trypan blue in the plasma, established by paper electrophoresis examination of plasma taken 30 hours after the last injection

The results thus show that if an intense vital staining is required for fairly short ... the intra-
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SUMMARY

In these experiments a programme for intraperitoneal injection of trypan blue was worked out by examining spectrophotometrically the disappearance rate of trypan blue in the plasma of the white rat

The material consisted of 7 rats It was found that injection of one millilitre of one per cent aqueous solution of trypan blue per 100 mg body weight at 24 hours intervals for 6 days produced no evident signs of toxicity There was, however, an accumulation of the stain in the plasma, and electrophoresis tests showed much of it to be in the free state

REFERENCES

Auskaps A M & Shaw J H Vital staining of calcifying bone and dentin with trypan blue J Dent Res 34 452 1955
Cappell D F Intravitam and supravital staining J Path Bact. 32 595 1929
Gregersen M I & Rawson R A The disappearance of T 1824 and structurally related dyes ...
Rawson R A ... 1943
plasma pr ... azo dyes by the
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... Leibniz Verlag Munich 1948

with sample cells having a light path of 5 cm The zero was adjusted against distilled water On *adding 10 µl of unstained plasma to 3 ml of distilled* water no measurable absorption values could be observed

From the results of this experiment the optimum interval between intraperitoneal injections was taken as 24 hours In the second experiment one injection was given daily for 6 days in succession to each of 4 rats Following the first and last injections samples were taken during the next 24 hours according to the schedule of the previous experiment On each of the 4 intervening days one sample was taken immediately prior to the injection In other respects the method was as for the previous experiment

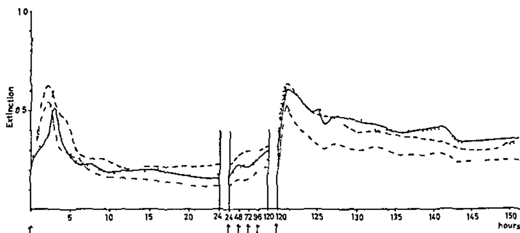


Fig 1

Relationship between time and extinction for plasma in 4 rats receiving one intraperitoneal injection of one per cent aqueous trypan blue on 6 days in succession The arrows mark the various times of injection

RESULTS

The results obtained during the first 24 hours of the second experiment, which are reproduced graphically in Fig 1, were similar to the corresponding results in the first In all of the animals the concentration of trypan blue in the plasma reached its maximum after about 2 hours The concentration fell sharply during the following two hours, and after 10 hours it had reached a value which thereafter diminished very slowly After 12 days there was still a detectable amount of trypan blue in the plasma

On repeated injections at 24-hour intervals the trypan blue in the plasma increased steadily Comparison of the extinction curves shows that the increase in concentration after the last injection was less than the corresponding increase after the first injection, and that after reaching its maximum value the concentration fell slowly and more steadily

The 4 animals suffered no ill effects from the administration of trypan blue and, at the end of the experiment, they were deep blue in colour

DISCUSSION

The resorption of dye from the sites of deposit evidently take place quite rapidly The curve for a single injection after the maximum con-

centration had been reached is similar to the one after intravenous injection reported by *Gregersen & Rawson* (1943) and *Anskops & Shaw* (1955) which, however, fell a little more rapidly The marked drop in the amount of trypan blue in the plasma during the first few hours after the maximum concentration has been reached has been ascribed to the type of bond between the dye and the plasma protein (*Rawson* 1943 *Gregersen & Rawson* 1943), whereby the trypan blue is linked primarily to the plasma albumin and only two molecules of the dye are linked to each albumin molecule Any excess of trypan blue will then be bound to the plasma globulins Since the capacity of the plasma protein to combine with trypan blue is limited, if the excess of dye is large part of it will remain free in the plasma and disappear rapidly from the circulation into the tissues and the urine It is this penetration into the tissues that accounts for the good staining properties of trypan blue

Because of the low toxicity of trypan blue and the fact that the plasma concentration falls slowly after 10 hours, the interval between injections could be reduced from the proposed 2–5 days to, say, 24 hours The continuous increase in plasma concentration in the 24-hour tests appears to be due to an increase in the amount of free trypan blue in the plasma established by paper electrophoresis examination of plasma taken 30 hours after the last injection

The results thus show that if an intense vital staining is required for fairly short experimental periods a suitable interval between the intraperitoneal injections is 24 hours Longer periods involve a risk of toxic action due to accumulation of free trypan blue in the plasma

SUMMARY

In these experiments a programme for intraperitoneal *injection of* trypan blue was worked out by examining spectrophotometrically the disappearance rate of trypan blue in the plasma of the white rat

The material consisted of 7 rats It was found that injection of one millilitre of one per cent aqueous solution of tr[illegible]

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Romeis B [illegible] lag Munich 1948

FROM THE MEDICAL DEPARTMENT, BORAS HOSPITAL, BORAS, SWEDEN (HEAD: OLOF FORSSMAN, M.D.)

INTERFERENCE BETWEEN ACTIVE PREPARATIONS OF INFLUENZA A AND INFLUENZA B VIRUSES

By

OLOF FORSSMAN

Received 30.ix.62

When influenza virus is injected into the allantoic cavity of an embryonated egg the propagation of another influenza virus in the allantoic membrane is prevented or partially inhibited (1, 2). Analyses of this phenomenon, which is termed interference, have contributed to a better understanding of virus reproduction, particularly when short intervals between the inoculation of the first and second virus are employed (3, 4). On the basis of the adsorption curves of the viruses used (5), and knowledge of the number of allantoic cells (6), the average number of virus particles adsorbed per cell at a given moment can be estimated. The interpretation of experimental data is facilitated by the calculation of the particle-cell ratio and it has been utilized in the present study.

This report deals with temporal and quantitative factors affecting the interference between fully active preparations of influenza A (PR8) and influenza B (Lee) viruses when large amounts are inoculated. It will be shown that the larger the number of infective virus particles inoculated, *the shorter is the time required before insusceptibility develops to the challenge virus.* It will be demonstrated that, under certain experimental conditions, active virus is capable of interrupting the reproductive process of heterologous influenza virus, already adsorbed onto the cells. Moreover, it will be shown that this capacity as well as the interfering capacity of infective preparations of Lee is far greater than that of PR8.

MATERIALS AND METHODS

Viruses.—The PR8 strain of influenza A and the Lee strain of influenza B virus were employed. Both viruses had been passed many times in the allantoic sac. Seed virus was prepared by inoculation of 0.2 ml of a 10^{-4} dilution of infected allantoic fluid into the allantoic cavity of 10 or 11 day old embryonated chicken eggs. The inoculated eggs were incubated at 35° C for 24 hours with PR8 and 36 hours with

This investigation was carried out at the *Rockefeller Institute for Medical Research*, New York, at the suggestion of the former chief of the virus department Dr. *Frank Horsfall Jr.*, who followed my work with keen interest and offered constructive criticism and valuable advice throughout the investigation. *For all this I* tender my sincere gratitude.

Lee so as to obtain fully infective preparations (5) Pools of allantoic fluid containing each virus were stored at —60° C

Buffered saline—Sodium chloride 0 85 per cent, buffered at pH 7 2 with 0 01 *M* phosphate was used

Red Blood Cells—Venous blood was drawn from normal roosters, mixed with acid citrate dextrose solution (7), pooled, and stored at 4° C for not more than 5 days Before use the red blood cells were washed 3 times in buffered saline and 0 5 per cent suspensions were prepared The concentration was calculated from the packed cell volume

Immune sera—Immune sera against PR8 or Lee were prepared in rabbits by intravenous injection of 10 ml of infected allantoic fluid followed by an intraperitoneal injection of 10 ml of similar fluid 2 to 3 weeks later Serum was obtained 2 to 3 weeks after the second injection and was stored at 4° C without preservative Before use sera were treated to remove chicken RBC agglutinins and virus inhibitor The RBC agglutinins were removed by adding packed chicken RBC to undiluted serum inactivated at 56° C for 30 minutes to give a 10 per cent RBC suspension in the serum The mixture was kept at 4° C overnight The RBC were

[illegible]

... of the fluid were made in buffered saline in volumes of 0 6 ml containing immune serum in a concentration sufficient to neutralize all haemagglutinating particles of the homologous virus Then 0 6 ml of 0 5 per cent ... [illegible]

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To determine the EI ... heterologous immune s... dilution tube to give ... preparation was added ... of immune serum was enough to prevent multiplication of the heterologous virus when injected into the allantoic sac Otherwise the procedure was the same as that described above

Computation of number of non-infective haemagglutinating particles—The number of non infective particles N was determined from the relation $N = H - I$ when H = haemagglutinating particles and I = infective particles as described previously (8)

Number of allantoic membrane cells—The number of cells lining the allantoic cavity of the 10 day old chicken embryo has been estimated to be $1\ 8 \times 10^7$ standard deviation $0\ 1 \times 10^7$ (6) This value was used for computation of virus particle allantoic cell ratios

Experimental procedures—The interference phenomenon was studied by two different procedures described below

1 *Threshold procedure*—A number of embryonated eggs were injected intra allantoically with one virus PR8 or Lee, and groups of 6 were reinoculated after various periods of incubation with the other virus Controls inoculated with one virus received saline as the other injection The eggs were incubated at 35° C for 18 to 28 hours which was sufficien
ing particles in the allantoic fluid
and tested individually Allantoic
was placed in test tubes which co
rabbit serum respectively The sera were prepared and treated as described above and diluted initially 1 125 which gave enough antibody to prevent haemagglutination by the homologous virus Chicken RBC, 0 4 ml of 0 5 per cent suspension were added to each tube giving a final dilution of 1 160 for the allantoic fluid The *readings were made after 1 hour*

2 *Quantitative procedure*—A group of 30 embryonated eggs was inoculated with one virus PR8 or Lee, and after the desired period of incubation with the other virus An equally large group of embryonated eggs served as controls for each of the viruses, in these saline was injected instead of the second virus All the inoculated eggs were incubated at 35° C and groups of 6 were removed from the incubator at 4, 12 16 20, and 24 hours after the second injection After storage at —26° C for 1 hour, the allantoic fluids from each groups were pooled The infectivity and the haemagglutination titer of each virus was determined with each pool as described above The geometric mean of the titers obtained at 16 20, and 24 hours was used in some analyses

The threshold procedure showed whether the haemagglutinating titer of either virus in each individual allantoic fluid was above or below 1 160 The quantitative procedure gave more precise information on the actual infectivity and heamagglutinating titers of each virus in the pooled allantoic fluids from each group of eggs

EXPERIMENTAL

Fully infective seeds viruses—To make the results of experiments on the kinetics of interference as unequivocal as possible it was desirable to use fully infective seed virus preparations as inocula The production of such influenza virus preparations was achieved by procedures described previously (5) The results of analysis of four PR8 and three Lee seed preparations used as inocula in this study are given in Table 1 The techniques employed for the enumeration of infective particles and hemagglutinating particles were identical with those used previously (8)

The ratio of the number of infective particles, I, to the number of haemagglutinating particles, H, was in the neighbourhood of 1 0 with each of the seven preparations On the basis of the errors inherent in the enumeration procedures used (5) the data support the view that each of the seed virus preparations could be considered as fully infective

In the experiments described below, both the number of virus particles inoculated and the virus particle-allantoic cell ratios, P C, were computed from the number of infective particles in the inocula

Experiments Utilizing the Threshold Procedure

Lee inoculated before PR8—A series of interference experiments was carried out in which large amounts of Lee virus were inoculated

at various intervals before inoculation with large amounts of PR8 virus The threshold procedure, described above, was employed and the allantoic fluid from each egg was tested at a final dilution of 1.160 for haemagglutinating of either type The objective was to determine the length of the interval after inoculation with Lee needed to produce interference with the multiplication of PR8

TABLE 1

Fully Infective Seed Virus Preparations of Influenza A (PR8) and Influenza B (Lee) Viruses

Virus	Infective particles No injected per egg	Incubation 35° C Hours	Allantoic fluid pool Infective particles (I) per ml	Haemagglutinating particles (H) per ml	Ratio I H
			× 10^7	× 10^9	
PR8	5 × 10^4	24	2 51	2 04	1 23
PR8	5 × 10^4	24	2 34	1 66	1 41
PR8	5 × 10^4	24	2 82	2 09	1.35
PR8	5 × 10^4	24	2 63	2 40	1 10
Mean			2 57	2 02	1 27
Lee	8 × 10^4	36	4 46	7 14	0 62
Lee	8 × 10^4	36	4 07	4 17	0 98
Lee	8 × 10^4	36	3 16	3 89	0 81
Mean			3 89	5 07	0 77

The results are presented in Fig 1 The number of infective particles of each virus inoculated at the indicated time is given as the ratio, P C, of virus particles to allantoic cells The ratios, H/T, of the number of eggs which were positive in haemagglutination tests for each virus to the total inoculated are plotted against the time between the inoculation of the first virus, Lee, and the second, PR8 When large numbers of Lee virus particles were inoculated, *i e*, P C *ratios ranging* from 83 to 3, the time needed to induce interference with a constant large number of PR8 virus particles, *i e*, P C ratio = 16, increased moderately as the number of the Lee particles was decreased The shortest interval observed was less than one hour with a Lee P C ratio of 83, and the longest was about 2½ hours with a P C ratio of 3 The time needed after the largest amount of Lee, remained about one hour even when the number of PR8 particles was increased to give a P.C *ratio of 83 In all* experiments there was a positive haemagglutination for Lee virus in all eggs, *i e* a haemagglutinating titer of more than 1 160

From these results, it appears that the time required for Lee *to induce* a state of relative insusceptibility to infection with PR8 is not strictly constant even when the number of Lee virus particles inoculated is

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1 *Threshold procedure*—A number of embryonated eggs were injected intra allantoically with one virus PR8 or Lee and groups of 6 were reinoculated after various periods of incubation with the other virus Controls inoculated with one virus, received saline as the other injection The eggs were incubated at 35° C for 18 to 28 hours which was sufficien ... inat- ing particles in the allantoic fluid ... hour and tested individually Allantoic ... 2 ml was placed in test tubes, which co ... ormal rabbit serum respectively The sera were prepared and treated as described above and diluted initially 1 125 which gave enough antibody to prevent haemagglutina tion by the homologous virus Chicken RBC 0 4 ml of 0 5 per cent suspension were added to each tube, giving a final dilution of 1 160 for the allantoic fluid The readings were made after 1 hour

2 *Quantitative procedure*—A group of 30 embryonated eggs was inoculated with one virus PR8 or Lee and after the desired period of incubation with the other virus An equally large group of embryonated eggs served as controls for each of the viruses, in these saline was injected instead of the second virus All the inoculated eggs were incubated at 35° C and groups of 6 were removed from the incubator at 4, 12, 16 20 and 24 hours after the second injection After storage at —26° C for 1 hour, the allantoic fluids from each groups were pooled The infectivity and the haemagglutination titer of each virus was determined with each pool as described above The geometric mean of the titers obtained at 16 20 and 24 hours was used in some analyses

The threshold procedure showed whether the haemagglutinating titer of either virus in each individual allantoic fluid was above or below 1 160 The quantitative procedure gave more precise information on the actual infectivity and heamagglu- tinating titers of each virus in the pooled allantoic fluids from each group of eggs

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In the experiments described below, both the number of virus particles inoculated and the virus particle allantoic cell ratios, P C, were computed from the number of infective particles in the inocula

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Lee inoculated before PR8—A series of interference experiments was carried out in which large amounts of Lee virus were inoculated

at various intervals before inoculation with large amounts of PR8 virus The threshold procedure, described above, was *employed and the* allantoic fluid from each egg was tested at a final dilution of 1:160 for haemagglutinating of either type The objective was to determine the length of the interval after inoculation *with Lee needed to produce* interference with the multiplication of PR8

TABLE 1

Fully Infective Seed Virus Preparations of Influenza A (PR8) and Influenza B (Lee) Viruses

Virus	Infective particles No injected per egg	Incubation 35° C Hours	Allantoic fluid pool		
			Infective particles (I) per ml	Haemagglutinating particles (H) per ml	Ratio I:H
			× *10*⁹	× *10*⁹	
PR8	5 × 10⁴	24	2.51	2.04	1.23
PR8	5 × 10⁴	24	2.34	1.66	1.41
PR8	5 × 10⁴	24	2.82	2.09	1.35
PR8	5 × 10⁴	24	2.63	2.40	1.10
Mean			2.57	2.02	1.27
Lee	8 × 10⁴	36	4.46	7.14	0.62
Lee	8 × 10⁴	36	4.07	4.17	0.98
Lee	8 × 10⁴	36	3.16	3.89	0.81
Mean			3.89	5.07	0.77

The results are presented in Fig 1 The number of infective particles of each virus inoculated at the indicated time is given as the ratio, P/C, of *virus particles to allantoic cells The ratios, H/T, of the* number of eggs which were positive in haemagglutination tests for each virus to the total inoculated are plotted against the time between the inoculation of the first virus, Lee, and the second, PR8 When large numbers of Lee virus particles were inoculated, *i e*, P/C ratios ranging from 83 to 3, *the time needed to* induce interference with a constant large number of PR8 *virus particles, i e*, P/C ratio = 16, *increased* moderately as the number of the Lee particles was decreased The shortest interval observed was less than one hour with a Lee P/C ratio of 83, and the longest was about 2½ hours with a P/C ratio of 3 The *time needed after the* largest amount of Lee, remained about one hour even when the number of PR8 particles was increased to give a P/C ratio of 83 In all experiments there was a positive haemagglutination for Lee virus in all eggs, *i e* a haemagglutinating titer of more than 1:160

From these results, it appears that the time required for Lee to induce a state of relative insusceptibility to infection with PR8 is not strictly constant even when the number of Lee virus particles inoculated is

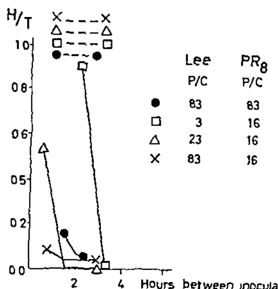


Fig 1

Number of eggs in which the haemagglutinating titer of either virus in the allantoic fluid was above 1 160 In each series of experiments 6 eggs were used Influenza B was inoculated before influenza A (PR8)
Fully infective virus particles were used as inocula

P/C = number of infective particles per allantoic membrane cell
H/T (Y axis) = Relative number of eggs which were positive in haemagglutinating tests for each virus
Solid lines = PR8 Dotted lines = Lee
The degree of interference was considered to be significant for values of H/T ≦ 0 5

large relative to the number of allantoic cells The amounts of Lee virus given were sufficiently large so that all or nearly all allantoic cells were infected before PR8 was injected

PR8 inoculated before Lee—A similar series of interference experiments was carried out in which large amounts of PR8 virus were inoculated at various intervals before inoculation of a large quantity of Lee virus The threshold procedure was also employed in this series and was identical with that described above The objective was to determine the time needed after inoculation with PR8 virus to induce interference with the multiplication of Lee virus

The results are shown in Fig 2b The number of infective particles of each virus inoculated and the ratios, P/C, of virus particles to allantoic cells are given As in Fig 1, the ratio, H/T, of eggs which showed haemagglutinating virus of either type relative to the total number of eggs is plotted against the time between inoculation of the first virus, PR8, and the second, Lee As the number of PR8 particles inoculated first was decreased to give P C ratios ranging from 83 to 16, and the number of Lee particles inoculated later was held constant, *i e*, P C = 16, the time required to induce interference increased The shortest time needed, about 1¾ hours, was found after giving PR8 at the highest P C ratio, *i e*, 83 The longest time needed, about 5½ hours, was found after inoculating PR8 at the lowest P C ratio, *i e* 16

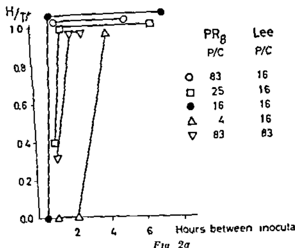


Fig 2a

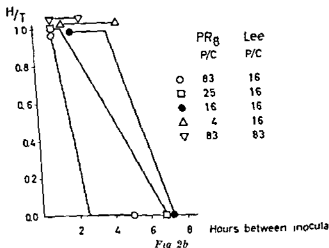


Fig 2b

Fig 2a and 2b

Number of eggs in which the haemagglutinating titer of PR8 (Fig 2a) and of Lee (Fig 2b) in the allantoic fluid was above 1 160 In each series of experiments 6 eggs were used PR8 was inoculated before Lee
Fully infective particles were used as inocula

P/C — number of infective particles per allantoic membrane cell
H/T (Y axis) — Relative number of eggs which were positive in haemagglutinating tests
The degree of interference (fig 2b) was considered to be significant for values of H/T ≦ 0.5

Thus it appears that when large amounts of PR8 are inoculated the time required to induce a state of relative insusceptibility to a large amount of Lee is not constant and increases as the P C ratio of the first virus is decreased from 83 to 16 These results are from a quantitative point of view considerably different from those secured when Lee was

the first virus inoculated It is noteworthy that when comparable amounts of virus were inoculated the time required for PR8 to induce interference against Lee was *considerably longer than that needed for* Lee to induce interference against PR8 Moreover, the increase in the time relative to reduction in the P/C ratio was much more marked with the PR8-Lee system than with the Lee-PR8 system

Interruption of PR8 Multiplication by Later Inoculation of Lee

During the interference experiments described above, in which PR8 virus was inoculated before Lee virus, certain unexpected results were obtained It was observed that when the number of virus particles of PR8 inoculated corresponded to a P/C ratio of 25 or less, later inoculation of Lee at P/C ratio of 16 sometimes interrupted multiplication of the first virus, PR8

The results of a series of experiments on the interruption of PR8 multiplication by later inoculated Lee are shown in Fig 2 a With a P/C ratio of 16 for both Lee and PR8, complete interruption was obtained (H/T = 0 0) when Lee was given ½ hours after PR8 When the amount of PR8 was reduced to give a P/C ratio of 4, interruption was demonstrated when Lee was inoculated as late as 2 hours after PR8 At this time 78 per cent of the PR8 inoculated had been adsorbed (8), and there can be little doubt that almost all susceptible cells were actually infected by PR8 Despite the late inoculation of Lee at a P/C ratio of 16 the multiplication of PR8 was interrupted and multiplication of Lee occurred It is noteworthy that a significant degree of interruption of PR8 multiplication (H/T = 0 3) did occur when as many as 83 PR8 particles per cell were injected one hour prior to 83 Lee particles per cell

Experiments Utilizing the Quantitative Procedure

The threshold procedure used in the experiments described above provided valuable information about the kinetics of interference and revealed that interruption of multiplication of PR8 could be induced by later inoculated Lee virus The threshold procedures have certain limitations showing merely whether the haemagglutination titer of either virus in the allantoic fluid is 1 160 or greater On the other hand, the quantitative procedure has the advantage that it gives definite values for haemagglutination and infectivity, and so provides more precise data on the kinetic aspects of interference This procedure was used in a series of experiments, described below The results obtained by the threshold procedure were of sufficient interest to warrant further efforts to analyze the dynamics of the reproduction of one type of influenza virus as affected by the other

Lee inoculated before PR8 —A series of interference experiments was performed in which Lee virus, at a P/C ratio of 23, was inoculated at

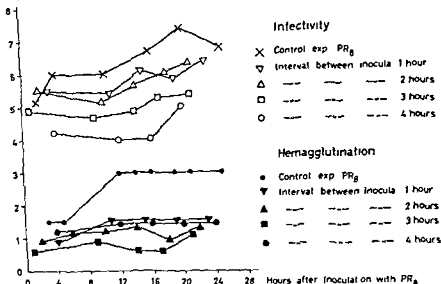


Fig 3

Infectivity and haemagglutinating titers of PR8 in interference experiments in which Lee was injected prior to PR8 Number of particles of either virus inoculated was 23 The corresponding values in control experiments are included in which saline was injected instead of Lee virus Each point is the result obtained after pooling of the allantoic fluids of 6 eggs

various intervals before inoculation of an equal amount of PR8 virus Because of the amount of virus inoculated it is highly probable that all of the allantoic cells had adsorbed Lee before PR8 was introduced The quantitative procedure, described above, was employed and the PR8 titer of pooled allantoic fluids from groups of eggs was measured at various periods after inoculation of PR8 virus Anti-Lee serum was added to each dilution of allantoic fluid before either infectivity or haemagglutination titrations were carried out The objects of these experiments were to determine the time needed for Lee virus to induce interference with the multiplication of PR8 virus and the degree of interference obtained when the interval between the two inocula ranged from 1 to 4 hours

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virus For purposes of analysis it was considered that mean titers 20 per cent or less for comparable control values were dependable indicators that interference had occurred As judged by both the infectivity and haemagglutination titers at 16 to 24 hours, interference with PR8 multiplication was induced as early as 1 hour after inoculation of Lee

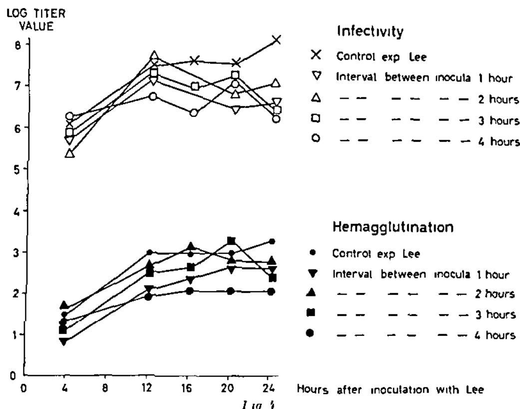


Fig 5

Infectivity and haemagglutinating titers of Lee in interference experiments in which PR8 was injected prior to Lee Number of particles of either virus inoculated was 23 The corresponding values in control experiments are included in which saline was injected instead of PR8 virus Each point is the result obtained after pooling of the allantoic fluids of 6 eggs

at a P/C ratio of 23 This corresponds with results obtained by the threshold procedure in experiments described above

As measured by the yield of infective PR8 virus, the extent of interference was more marked for an interval between inocula of 3 hours than of 1 of 2 hours However, haemagglutination titers indicated that interference was almost as extensive at 1 hour as at the later intervals It is evident, too, that in no experiment was the multiplication of PR8 wholly prevented Despite the inoculation of Lee at a P/C ratio of 23,4 hours before inoculation of PR8, both the infectivity and haemagglutination titers indicated that some multiplication of PR8 occurred even though the yield was not more than 3 per cent of the control values

PR8 inoculated before Lee —Another series of interference experiments was carried out in which PR virus, at a P/C ratio of 23, was inoculated at various intervals before inoculation of an equal amount of Lee virus In these experiments all of the allantoic cells undoubtedly had adsorbed PR8 before Lee was introduced The quantitative procedure was employed and the Lee titers of pooled allantoic fluids from groups of eggs were measured at intervals after the inoculation of Lee virus Anti-PR8 serum was added to each dilution of allantoic fluid before infectivity or haemagglutination titrations were carried out

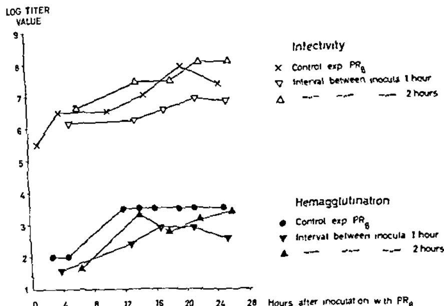


Fig 5

Infectivity and haemagglutinating titers of Lee in interference experiments in which PR8 was injected prior to Lee Number of particles of either virus inoculated was 23 The corresponding values in control experiments are included in which saline was injected instead of Lee virus Each point is the result obtained after pooling of the allantoic fluids of 6 eggs

The results are shown in Fig 4 The infectivity and haemagglutination titers of Lee virus in each experiment are compared with those obtained in control eggs previously injected with saline instead of with PR8 virus On the basis that mean titers 20 per cent or less of comparable control values were dependable indicators of interference, the haemagglutination titrations at 16 to 24 hours showed that an interval of 4 hours was needed before PR8 induced interference with the multiplication of Lee The infectivity titers were more variable but the mean titers at 16 to 24 hours indicated that a moderate degree of interference, *i e* about 8 per cent of the control values, was induced at an interval of only 1 hour However, the amount of interference did not increase appreciably as the interval between the inoculations was increased to 4 hours

The results obtained with haemagglutination titrations correspond fairly well with those described earlier using the threshold procedure (cf Fig 2b) It seems clear that PR8 did not induce interference against Lee virus multiplication either as rapidly or as effectively as did Lee virus against PR8 multiplication It is noteworthy that in spite of the large inocula employed, *i e*, PC ratio = 23, and the length of the intervals between inocula, some multiplication of Lee virus occurred in

every experiment The smallest mean yield was about 8 per cent of that in the controls

Interruption of PR8 Multiplication by Later Inoculation of Lee

In another series of experiments by which to determine if later inoculated Lee virus would interrupt the multiplication of PR8 virus, similar amounts of the two viruses were employed, *i e*, P/C ratios of 23 The quantitative procedure was used, as in the experiments described above, and anti-Lee serum was added in each titration so that the amount of *PR8* virus could be estimated The results are shown in Fig 5 The infectivity and haemagglutination titers of PR8 are compared with those in control eggs which did not receive Lee virus Mean infectivity and haemagglutination titers of PR8 at 16 to 26 hours were 20 per cent or less of control values, when Lee was inoculated 1 hour after PR8 However, when inoculation of Lee was delayed until 2 hours, *the reduction in the PR8 titers was not of significant degree* These findings are in accord with those secured with comparable inocula in experiments with the threshold procedure (cf Fig 2 a) and indicate that when all cells have adsorbed PR8, later inoculated Lee can interrupt PR8 multiplication to a significant degree when given 1 hour after PR8

Failure of PR8 to Interrupt Multiplication of Lee Previously Inoculated

Experiments were performed identical to those preceding, excepting only that PR8 virus was introduced after Lee and the titers achieved by Lee were determined *It was clearly shown that PR8 (P/C ratio = 23)* was not capable of interrupting the multiplication of Lee (P C ratio = 23) even when introduced as early as 1 hour after Lee It will be recalled that similar experiments with the threshold procedure also failed to show any evidence that later inoculated PR8 could interrupt Lee virus multiplication —Analysis of the titers of PR8 in this series of experiments may, however, further clarify this problem (cf *Discussion*)

Attempts to Demonstrate the Presence of Combination Forms of Virus Particles in the Experiments Described

Simultaneous inoculation of two different serological types of influenza strains into the allantoic cavity can result in the formation of combination forms of virus which contain antigens derived from both parent types (9) Such particles are neutralized by both antisera and are accordingly demonstrated by the finding of a haemagglutinating titer of a mixture of virus strains that is higher than the sum of the titers of individual components

In the experiments described above, the total haemagglutination titer of the yield was not determined However, in all experiments the titer of at least the *one* kind of virus came close to the corresponding titer

obtained in the control eggs which did not receive the heterologous virus

Hence it is unlikely that the inoculations of PR8 and Lee gave rise to any such combination types of virus

DISCUSSION

The main work on interference in the allantoic membrane of the chick embryo system between type A and type B strains of influenza virus has been done on interference by inactivated agents (For reviews see 10 12) Analyses of results obtained in such a system have greatly contributed to a better understanding of virus multiplication However, such experimental conditions are limited to studies of reproduction of only one kind of virus and for obvious reasons not of both In the present investigation fully active virus particles were used in *both* inocula making it also possible to observe any influence of the second virus on the replication of the first virus The procedure of using active virus preparations eliminated the possibility of particles interfering with the propagation of the active agent present in the same preparation Moreover the experimental conditions were selected in such a way that all cells were undoubtedly provided with virus particles of both kinds within the latent period of the first virus This made it possible to avoid the uncertainty of interpreting results obtained from multiple reproduction cycles

Further analysis on the events of virus reproduction in the allantoic membrane were based on 1) the lack of so called combination forms of PR8 and Lee in the actual experiments and 2) the assumption that new particles of one kind of virus but not of both can be released from one and the same cell

Therefore since all cells were blocked by previously injected virus any propagation of the second virus even to a small extent strongly indicates a mutal exclusion of the "interfering agent in *some* cells The finding of a reduced increment of the later inoculated virus is usually termed interference and is obvious the adequate explanation of the event occurring in the *remaining* cells Thus it can be concluded that there are qualitative differences in various cells regarding the reproductive processes at a given moment These variations may partly be explained on the basis of differences in distribution of virus particles to various cells both during and after adsorbtion The most striking findings to emerge from this investigation are however that other factors also play a part of reflecting a difference in the properties of PR8 and Lee

Reciprocal interference experiments thus revealed that the multiplication of the second virus became significantly more suppressed in the system Lee PR8 than in the system PR8 Lee Hence Lee virus was found to be a far more powerful agent than PR8 virus in preventing

the multiplication of active heterologous virus injected in advance In one instance in which PR8, at a particle-cells ratio of 16, was inoculated before inoculation of an equal amount of Lee, multiplication of Lee, although slight, was found to occur even when the interval between injections was *extended to 5½ hours (Fig 2b)* This observation implies that Lee virus was capable of interrupting the reproductive process of PR8 in *some* cells throughout almost the whole latent period of PR8

The existence of such a quantitative difference between the properties of PR8 and Lee was supported by another and more direct approach, namely by comparing the yield of the first virus in interference experiments with controls, where saline was given instead of the second virus With the virus dosis and intervals between inocula described, significant reduction of the infectivity and the haemagglutination titer of the first virus only occurred when PR8 was injected prior to Lee, but not vice versa This reduction may be partly explained by the fact that the total number of virus particles injected (1st *and* 2nd inoculum) was higher in the experimental series than in the controls Damage to the cells of the allantoic membrane may have occurred when a higher number of particles per cell were adsorbed, followed by a decrease in the efficiency of the reproductive processes (5, 8) This hypothesis, however, cannot explain the difference observed in reciprocal experiments and the statement on the interruption of the first virus achieved by the analysis of the yield of the second virus

Conclusive evidence of an interruption of the reproduction of the first virus, caused by the second virus, has not been previously presented for active preparations of influenza virus Earlier observations, bearing on this problem (2), may be interpreted in various ways since only small inocula of *either virus were used, i e less than one virus particle per* cell

Naturally, other results may be obtained in interference experiments dealing with inactivated virus which, at least partly, has another action on the cells than live virus Thus, inactivated influenza virus induces *the allantoic cells to produce interferon in contrast to active virus, at* least while the cells are fully engaged in virus production (12) It may be mentioned, however, that experimental data indicating that irradiated virus is capable of reducing the titer of active virus injected *in* advance, may as well have alternative explanations Thus, in some series only a small dose of active virus (< 1 particle per cell) was used (13, 14) and the findings may then be explained as resulting from the inhibition of a second multiplication cycle of the active virus, caused by the second virus In other series (15 18) the results may instead be interpreted as the production of "incomplete" virus (19) It can therefore be said that unequivocal evidence has not previously been produced that influenza virus, active or inactive, is capable of interrupting the multiplication of active heterologous or homologous virus inoculated in advance

The stronger capacity of Lee, compared with PR8, in preventing the multiplication of active heterologous virus, injected before, corresponds to the greater interfering capacity of Lee than of PR8 which was clearly demonstrated in this study It seems reasonable to assume that these two properties, interference, and interruption of the multiplication of the first injected virus, are merely two aspects of the same process It is evident that, ultimately, they both imply a reproduction at cellular level, of particles of one kind and prevention of the reproductive process of the other

SUMMARY

Interference of fully active seeds of influenza A (PR8) with influenza B (Lee), and vice versa, was studied in the chick-embryo system for relatively short intervals between inoculations All the experiments were designed in such a way, that, when the second inoculation was performed, all allantoic cells were already infected with one or more particles of the virus introduced as the first agent By making the second inoculation sufficiently large, it was ensured that all reproductive and interfering processes occurred during the first cycle of reproduction

The degree of interference was estimated either on the basis of infectivity and haemagglutinating titers in the allantoic cavity, or, depending on the method used, only on the haemagglutinating titers obtained in the actual experiment and compared with the corresponding values in control experiments

Primarily, the aim of the present study was to obtain information on temporal and quantitative factors that affect interference in the reciprocal systems PR8 – Lee and Lee – PR8 A comparison of results obtained for the two systems revealed qualitative similarities but quantitative differences Thus, in both systems, the interval between inocula, which was needed to induce interference with a constant dose of the heterologous virus (corresponding to a particle-cell ratio of 16), was not constant, but increased when the number of particles of the first

[illegible] PR8 in inducing interference

This difference between PR8 and Lee was repeatedly confirmed in other series of experiments where a different set-up was used

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of the first [illegible] that this process occurred in the allantoic membrane at the same time as the interference but in different cells Comparison between the capacity of PR8 and Lee to inhibit the reproduction of previously injected virus particles, revealed that Lee was predominant also in this respect In one instance, Lee virus was capable of interrupting multiplication of PR8 throughout almost the whole

the multiplication of active heterologous virus injected in advance In one instance in which PR8, at a particle-cells ratio of 16, was inoculated before inoculation of an equal amount of Lee, multiplication of Lee, although slight, was found to occur even when the interval between injections was extended to 5½ hours (Fig 2b) This observation implies that Lee virus was capable of interrupting the reproductive process of PR8 in *some* cells throughout almost the whole latent period of PR8

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SUMMARY

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Primarily the aim of the present study was to obtain information on temporal and quantitative factors that affect interference in the reciprocal systems PR8 – Lee and Lee PR8 A comparison of results obtained for the two systems revealed qualitative similarities but quantitative differences Thus in both systems the interval between inocula which was needed to induce interference with a constant dose of the heterologous virus (corresponding to a particle cell ratio of 16), was not constant but increased when the number of particles of the first virus was reduced In these reciprocal experiments however a striking difference was found between the two interfering agents in as much as Lee was more powerful than PR8 in inducing interference

This difference between PR8 and Lee was repeatedly confirmed in other series of experiments where a different set up was used

Another phenomenon besides *interference* was *sometimes observed* in these experiments viz the second virus inhibited the multiplication of the first virus It is likely that this process occurred in the allantoic membrane at the same time as the interference but in different cells Comparison between the capacity of PR8 and Lee to inhibit the reproduction of previously injected virus particles revealed that Lee was predominant also in this respect In one instance Lee virus was capable of interrupting multiplication of PR8 throughout almost the whole

latent period of PR8 The dose of PR8 and Lee was then the same, corresponding to a particle-cell ratio of 23

REFERENCES

1 *Henle W & Henle G* Interference of inactive virus with the propagation of influenza Science *98* 87, 1943

2 *Ziegler J E & Horsfall F L* Interference between the influenza viruses 1 The effect of active virus upon the multiplication of influenza viruses in the chick embryo J Exp Med *79* 361 1944

3 *Henle W* Interference phenomena between animal viruses a review J Immunol *64* 203 1950

4 *Henle W & Paucker K* *Interference between inactivated and active influenza* viruses in the chick embryo I A re evaluation of factors of dosage and timing using infectivity titrations for assay Virology *6* 181 1958

5 *Horsfall F L Jr* Reproduction of influenza virus Quantitative investigations with particle enumeration procedures on the dynamics of influenza A and B virus reproduction J Exp Med *102* 441 1955

6 *Tyrrell D A J Tamm I Forssman O C & Horsfall F L Jr* *A new count of* allantoic cells in the 10 day chick embryo Proc Soc Exp Biol Med *86* 594 1954

7 *Rapoport S* Dimensional osmotic and chemical changes of erythrocytes in stored blood I Blood preserved in sodium citrate neutral and acid citrateglucose (ACD) mixtures J Clin Inv *26* 591 1947

8 *Horsfall F L Jr* *On reproduction of influenza virus quantitative studies* with procedures which enumerate infective and hemagglutinating virus particles J Exp Med *100* 135 1954

9 *Hirst G K & Gotlieb T* The experimental production of combination forms of virus I Occurrence of combination forms after simultaneous inocula tion of the allantoic sac with two distinct strains of influenza virus J Exp *Med 98 41 1953*

10 *Henle W* Multiplication of influenza virus in the entodermal cells in the allantois of the chick embryo p 141 in Advances in Virus Research vol I ed by Smith K & Lauffer M Academic Press 1953

11 *Schlesinger W* Interference between animal viruses p 157 in The Viruses ed by Burnet M M & Stanley W M Academic Press 1959

12 *Isaacs A Viral interference in Virus growth and variation ed by Isaacs A* & Lacey B W The Ninth Symposium of the Society for General Micro biology 1959

13 *Ziegler J E Jr Lavin G J & Horsfall F L Jr* Interference between the influenza viruses II The effect of virus rendered non infective by ultra violet radiation upon the multiplication of influenza virus in the chick *embryo J Exp* Med *79* 379 1944

14 *Henle W & Henle G* Interference between inactive and active virus of in fluenza II Factors influencing the phenomena Am J Med Sci *207* 717 1944

15 *Henle W Henle G & Rosenberg E B* The demonstration of one step growth curves of influenza viruses through the blocking effect of irradiated virus *on further infection* J Exp Med *86* 423 1947

16 *Henle W & Rosenberg E B* One step growth curves of various strains of influenza A and B strains and their inhibition by inactivated virus of the homologous type J Exp Med *89* 279 1949

17 *Henle W* [illegible] virus syster [illegible] t cell J Ex[illegible]

18 *Paucker K & Henle W* Interference between inactivated and active influenza viruses in the chick embryo II Interference by incomplete forms of influenza virus Virology *6* 198 1958

19 *Liu O C Paucker K & Henle W* Studies on Host virus interactions in the chick embryo influenza virus system XIII Some Aspects of Non Infec tious Virus Production J Exp Med *103* 777 1956

AN OUTBREAK OF POLIOMYELITIS IN A PARTIALLY VACCINATED POPULATION

The Epidemic in Gothenburg in 1961

by

TRYGVE ANDÉN and ERIK LYCKE

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In 1953 poliomyelitis was epidemic in the greater part of Sweden including the western provinces and the city of Gothenburg. Vaccination with inactivated vaccine was initiated in these areas in 1957 and gradually all people below the age of 50 years were offered vaccination free of charge. Nevertheless, a local outbreak of poliomyelitis occurred in Gothenburg in 1961. As at this time the susceptible age groups of the poulation were vaccinated to a very high percentage, a study of the epidemic was considered to be of a particular interest.

EPIDEMIOLOGICAL BACKGROUND

During the summer of 1961 outbreaks of poliomyelitis were reported from Western Germany, Great Britain, and Denmark. More than 1,154 cases were observed in June and July, Table 1 (1, 2).

TABLE 1

Reported Cases of Poliomyelitis in Denmark, Federal Republic of Germany and Great Britain during June and July of 1961

	Number of cases in June 1961	Number of cases in July 1961
Denmark	–	60
Federal Republic of Germany	277	629
Great Britain	94	94
Total	371	783

Gothenburg, the main sea port of Sweden, is an industrial center with a little more than 400 000 inhabitants. The early number of new settlers is relatively large. For the years preceding 1961 the frequency of poliomyelitis in Gothenburg corresponds fairly well to the incidence

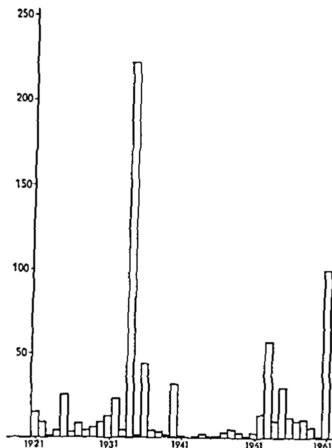


Fig 1
The number of reported cases of poliomyelitis in Gothenburg 1921–1961

reported for Sweden as a whole Figure 1 shows the reported number of cases, paralytic and aparalytic, in Gothenburg for the years 1921 to 1961 Since the large epidemic in 1953 till July, 1961, only 70 cases of poliomyelitis have been reported

TABLE 2
Percentage of Vaccinated Individuals in the Population Individuals Born in 1910 to 1960

Age groups (years of birth)	Percentage of population	
	receiving 3 injections	receiving 2 injections
1955–60	75	
1945 54	95	
1933 44	47	7
1921 32	41	15
1910 20		60

Table 2 shows the number of vaccinees in Gothenburg in per cent of the total number of individuals in each age group About 75 per cent of the pre-school children were vaccinated, the majority of these re-

ceived their injections at children welfare centers Of the school children 95 per cent received all three injections at school All children who received the first injection fulfilled the whole vaccination series A certain number of individuals born in the years 1921–1944 and the whole age group 1910–1920 were offered vaccination during March May, 1961 In addition to the campaign organized by the health authorities vaccinations were also performed by practitioners These vaccinations were, however, relatively few During the epidemic, i e from June till the end of December, no public vaccinations were performed

All the vaccine used was produced by the National Bacteriological Laboratory, Stockholm Three one ml injections were given, the interval between the first two injections was one month the third injection was given 8–12 months later

THE EPIDEMIC

The two first reported cases occurred on July 5, 1961 They were two girls 4 and 6 years old, playmates who lived in the same house Their fathers worked at the same factory as did also the husband of a 22 years old female who represented the third case Simultaneously a fourth case appeared the patient being an associate to the family of one of the girls Thus contact between these cases was demonstrable During the first 6 weeks of the epidemic the 26 cases reported were localized to one single district Later, cases occurred also in other districts of the city However many of these also appeared to have been in contact with the district in which the epidemic first started

In total 100 cases were registered The age distribution of these is illustrated in Fig 2 and 3 As shown, most of these were children of pre school ages Thus, 50 were less than 7 years old and 6 less than one year old The oldest patient was 49 years old, the youngest was a newborn girl (3) As mentioned the first cases were found on July 5 and the last case occurred on December 27, 1961

Fifty cases were diagnosed as paralytic poliomyelitis In 12 of the cases paresis of a transient nature was noted, including paresis of oculo motor of mimic, and peroneal muscles Thirty eight of the cases were of a paralytic nature the pareses persisting for more than two months Fifty additioned cases, were registered as aparalytic poliomyelitis The diagnostic criteria for aparalytic cases were meningitis with spinal fluid white cell counts of 5 cells per cubic millimeter of ...

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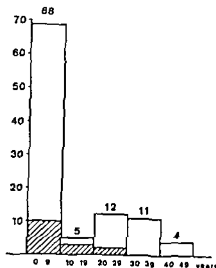


Fig 2
Age distribution of cases The shadowed part of the columns refer to paralytic cases

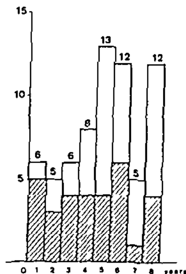


Fig 3
Age distribution of cases The shadowed part of the columns refer to paralytic cases

ping walk was demonstrable which persisted for one month upon which it disappeared In the other a facial paresis was recorded which disappeared within a few days and a paresis of n abducens which persisted for more than two months Thus, of 38 patients with paresis persisting for more than two months only one had received three vaccine injections

The district of the city in which the epidemic started is composed of an older part due for slum-clearance and blocks of modern apartment houses and industries A relatively large number of young families with many children are living here The percentage of vaccinated individuals in this district was not significantly inferior to figures shown in table 1 for the city as a whole If the number be considered of vaccinated indivi-

duals in the families, in which cases of poliomyelitis occurred, the figures, however, are lower than the average In total 302 family members were registered Of these 115 were vaccinated, *i e* 38 per cent, and among the children of pre-school ages less than one third The number of individuals are too small, however, to allow any conclusions

VIROLOGICAL EXAMINATIONS

Stool and blood specimens were collected from all of the cases The majority of the specimens were taken during the first week of disease, in a few cases, however, as late as one month after the onset of the disease The stool specimens were tested in monkey kidney and HeLa cell cultures Poliovirus type I was isolated from all of the cases Homologous neutralizing antibodies were demonstrable in all of the cases as well and complement fixing antibodies were found in 97 of the patients

From 9 patients diagnosed as aseptic meningitis, two type B2 and seven type B5 of Coxsackie viruses were isolated These patients were, however, not included in this study, as mentioned above

DISCUSSION

Comparison of attack rates between comparable vaccinated and unvaccinated groups represents an useful measure of poliovaccine efficacy Since the introduction of poliovaccinations in Sweden till the actual epidemic in Gothenburg the frequency of poliomyelitis has been too low to allow adequate comparisons of this kind In the town of Eskildstuna an outbreak of poliomyelitis in 1958 due to polio virus type I caused 35 paralytic cases none of which had been vaccinated completely with Swedish vaccine (4)

In U S A the efficacy of the Salk vaccine has been estimated by studies of *e g* the epidemic in Des Moines and Kansas City in 1959 (5 6) In Des Moines 135 cases occurred of which 70 were of a paralytic nature and in Kansas City 210 cases were registered including 118 paralytic cases A comparison between these two epidemics and the epidemic in Gothenburg presents some interesting similarities

The epidemics appeared in populations vaccinated to a very high percentage and were each strictly localized to a city The highest attack rates were found among children of pre school ages Thus in Des Moines the attack rate was found to be 1½ and in Kansas City 3 times higher than rates observed among the school children In Gothenburg the attack rate in the pre-school age-groups was also 3 times higher than among the schoolchildren In all three cities children in the school-age groups were found to be completely vaccinated Comparison between the pattern of the epidemics occurring before and after the poliovaccine era has in U.S A showed a marked trend towards higher rates among the pre-school children in epidemics occurring after the

introduction of the Salk vaccine than had hitherto been observed Regarding Gothenburg the pre-school children constituted 44 per cent of the total number of cases registered in the 1953 epidemic whereas the corresponding figure for 1961 was 50

In the American reports the high incidence of poliomyelitis among the lower socioeconomic groups is emphasized Although it will be difficult – considering the social structure of Sweden – to make similar observations here, the actual epidemic started, and the majority of the cases occurred in an area of the city in which housing conditions are of a less satisfactory standard

The total attack rate per 100,000 population calculated for the epidemic in Gothenburg was 24 7 The protective efficacy[1] of three vaccine injections measured on the basis of paralytic as well as of aparalytic cases in the age-groups below 50 years was 91 9 per cent If only the 38 cases involving paresis persisting for a period longer than two months were considered the vaccine effectivity to reduce paralytic polio was calculated to 98 3 per cent The vaccines used have thus proved to be highly protective and previous observations of the high immunizing efficiency of the Swedish poliovaccine have been confirmed

SUMMARY

The epidemiological pattern of an epidemic of poliomyelitis in Gothenburg in 1961 was studied One hundred cases were registered 50 of which were of a paralytic nature The attack rate per 100,000 population was 24 7 The attack rate was highest among children in the pre school age-groups and lowest among the school-children who were vaccinated to 95 per cent

Eleven of the affected individuals had received three injections with poliovaccine Of these patients one 5-year-old boy, only suffered from paresis persisting for a period longer than two months The vaccine efficacy was calculated to 98 3 per cent as regards reduction of paralytic polio and to 91 9 per cent if based on the figures for aparalytic as well as paralytic polio

REFERENCES

1 Epid Vital statist Rep *14* 219 1961
2 Epid Vital Statist Rep *15* 89 1962
3 *Lycke E & Nilsson L R* Poliomyelitis in a newborn due to intrauterine infection Acta Paediat *51* 1962 in press
4 ... *Margareta Kaijser K & Nässén N* A polio epidemic in ... ulation Fifth Internat Poliomyelitis Conference ... idelphia Montreal 1961
5 ... The changing pattern of poliomyelitis observed ... Health Rep *76* 553 1961
6 *Marine* ... *F* Sjergel Effectiveness of Salk v... a large urban epidemic New ...

1 Vaccine efficacy $= \frac{\text{Expected cases} - \text{observed cases}}{\text{Expected cases}}$

FROM THE TREPONEMATOSES DEPARTMENT, STATENS SERUMINSTITUT, COPENHAGEN

THE REITER STRAIN OF TREPONEMA PALLIDUM

Origin, Cultivation, and Use in Immunization of Rabbits

By

AA HEIN CHRISTIANSEN

Received 1 viii 62

ORIGIN

By reviewing the literature it has been possible to trace the so-called Reiter-treponeme until about 1922, when *Wassermann & Ficker* (17) reported that they had succeeded in isolating seven strains of *Treponema pallidum* from 80–90 primary cases of syphilis and culturing these strains in fluid and semifluid medium One of these isolated strains, B 36, was able to cause typical syphilitic lesions when inoculated into rabbit testes, and Ficker was able to regain B 36 from the infected testes making subcultures in vitro When *Ficker*, in 1923, left the Kaiser-Wilhelm Institute of Berlin, his work on B 36 was continued by *Reiter*, who isolated some new strains (B 49 and B 64), also from primary cases These strains, however, did not possess the virulence of B 36, since an orchitis was not always obtained as a result of injections of B 49 and B 64 (13, 14) *Reiter* sent treponemes to other investigators, among whom may be mentioned *Klopstock* (10), *Mulzer & Nothaas* (11), *Gaehtgens* (9), *Beck* (1), *Eagle & Germuth* (6) *Klopstock & Gaehtgens* prepared an antigen (so-called Palligen) from the Reiter strain for diagnostic purposes as to syphilis (9, 10), *Mulzer & Nothaas* were able to produce a specific rabbit orchitis using a Reiter strain, which had been passed through two hundred subcultures in a fluid culture medium It is uncertain whether this strain was the B 36, because the protocols have been lost (13) Beck found a [illegible]

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fore m [illegible] by agglutination and complement-fixation experiments, a cross-reaction was demonstrated between the Reiter and the Kazan strains showing that these were related, though not identical On the other hand, an almost complete identity was found between the Reiter and two strains of saprophytic mouthspirochaetes

From *Eagle*, the Reiter strain came back to Europe to *D'Alessandro & Dardanoni* in Italy (1953), and to Germany, where the strain was lost during the Second World War (13) Later *D'Alessandro* sent the Reiter strain to *De Bruijn & Bekker* in Holland and to the Statens Seruminstitut in Copenhagen When we lost this original strain, a new one was received from *De Bruijn* in 1959

From the above it is realized that this strain seems to be the B 36, or at least a strain which is serologically identical with this

The results of *Wassermann & Ficker* and *Mulzer & Nothaas* have never been reproduced, and thus are hard to understand and explain However, it is now generally accepted that the Reiter strain is apathogenic (18) *Eagle & Germuth* (6) state that the validity of identification of the Reiter-treponeme as *T pallidum* is questionable because it differs from the pathogenic one both as regards morphologic and antigenic properties

The possibility remains that the Reiter treponeme is derived by mutation from the pathogenic *T pallidum*, since it has antigenic similarities with the latter in the lipid and protein fractions

On the other hand, the Reiter treponeme may be a saprophytic skin spirochaete isolated, by accident, from a syphilitic primary case But the fact that the Reiter-treponeme is anaerobic, makes this hypothesis improbable

CULTIVATION

The Reiter treponeme varies in length between 6 and 44 microns the average being about 18 microns after 48 hours growth (15) In addition some ring forms and cysts may be found The motility of the treponemes in a fresh culture is very great approaching that of the pathogenic strains Contrary to the latter the Reiter treponemes possess the ability to survive for weeks in the culture medium in a vegetative stage where the motility is markedly reduced At microscopy all treponemes in such an old culture may apparently be dead but evenso they may start multiplication when placed in a fresh medium under proper conditions

CULTURE MEDIUM

The Reiter treponemes are grown in Brewer's thioglycolate medium (2) or in some modification of this In Copenhagen the following modification is used

Trypticase[1]	15 0 grams
L cystine	0 75
Glucose	5 0
Yeast extract (Difco)	5 0
NaCl	2 5
Na thioglycolate	0 5
Resazurin	0 001
Agar	0 75

Preparation of Medium

The cystine is dissolved in a few drops of 5 N HCl and the solution added to the water together with agar water soluble yeast extract trypticase and NaCl and

[1] trypsin digested casein

heated in a water bath until the dissolution is finished Then the thioglycolate is added and if necessary enough N NaOH to give a pH of 7 1 ± 0 1 after sterilization Simultaneously with the thioglycolate 1 ml of a freshly prepared 0 1 per cent solution of resazurin is added If filtration is necessary to clear the solution this shou
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sion of oxygen into the medium For mass cultivation the agar should be avoided because it is difficult to remove from the treponemes Besides agar is unnecessary here because
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ays at 37° C to check the sterility

INOCULATION AND GROWTH

The tubes are inoculated with about 1 ml of culture, the bottles with 5 ml Before inoculation the seed cultures are examined for contamination by inspection of a Gram stained preparation Contamination of the tubes is extremely seldom if common procedures in the bacteriological technique are followed The inoculation technique of the bottles is more complicated because the caps have to be unscrewed and replaced, and also the amount of inoculum is larger Thus the bottles must remain open for a longer period than the tubes during the inoculation and the inoculation should be performed under a hood fitted with ultraviolet light Also care should be taken that the glassware, and other things used should be thoroughly flamed just before use

If contamination of the seed cultures occurs it may be troublesome to obtain a clean culture again In this case, it may be helpful that the treponemes are able to grow on a common 5 per cent blood agar plate under anaerobic conditions (8)

After inoculation the tubes or bottles are incubated at 37° C and growth is visible already after 24 hours reaching an optimum after 4 5 days harvest should take place after 5 days During growth the treponemes settle in the bottles and these are therefore gently rotated once or twice each day to resuspend the organisms As growth proceeds a dark debris is formed the nature of which is uncertain Wallace & Harris (16) suggest that it may be metabolic endproducts of a medium component The amount of this material varies in an unpredictable way from time to time and has hitherto been impossible to separate completely from the organisms

The treponemes are separated from the medium by centrifugation at +4° C. This procedure may be facilitated by using a centrifuge with a so-called continuous flow system attached (for example M.S.E. High Speed 17 with refrigeration and continuous flow). The advantage of using a continuous flow is that the suspension is kept in a closed, sterilized system during the centrifugation, thus minimizing the risk of contamination. After centrifugation the treponemes are washed 3 times in saline to remove culture medium residue. If not used at once, the organisms can be stored in the lyophilized state. The average yield will then be 1.5 to 2 grams per 5 liters of medium (dry weight).

The above mentioned procedure kills the treponemes. However, if the treponemes are stored in the medium in sealed ampoules and kept in the dark at room temperature, they remain living in a vegetative state for at least 3 months. Although spontaneous movements are hardly seen by microscopy in a culture like this, growth occurs rapidly, when a subculture is prepared in fresh medium. Lyophilization seems to kill the treponemes, even if it is done from a broth culture.

IMMUNIZATION OF RABBITS

Preparation of Vaccine

Lyophilized treponemes are suspended in saline (with a content of phenol of 0.25 per cent by volume) giving a density corresponding to twice that of International Coli Standard 8. The dry weight content of this vaccine is 2.4 mg per ml. The vaccine is homogenized using a 10 ml syringe and a coarse needle, and stored in rubbercapped tubes at + 4°C.

The vaccine may be prepared in a similar manner avoiding density measurement by calculation of the needed amount according to the following immunization scheme and weighing a convenient amount of treponemes to be suspended in phenolized saline as prescribed.

Immunization scheme

Serum from male rabbits weighing about 3 kg is tested for reactivity in the Wassermann (CWRM), Kahn (KR) and Meinicke (MR) reactions. Only the negative animals are used. The vaccine is given in the ear veins in the following manner: First day 0.5 ml, third day 1.0 ml, sixth day 1.0 ml, and then 2 ml every second day until a total of 10 injections have been given. 7–8 days after the last injection, blood samples are drawn from the ear veins or by heart puncture.

Fig. 1 shows a typical progress of seroreactions of immunized rabbits when serum samples are tested against cardiolipin-antigen and Reiter polysaccharide-antigen (3, 4). Four rabbits have been immunized by six injections of lyophilized Reiter treponemes. The injections are indicated in the figure by arrows. Blood samples have been drawn simultaneously, and after the last injection, once a week for 6 weeks. The blood samples have been examined in complement-fixation tests with the

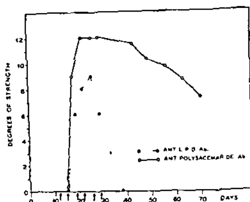


Fig 1

Antipolysaccharide and antilipid antibodies present in rabbit serum during and after immunization with lyophilized Reiter treponemes
The arrows indicate injection of treponemes

above two antigens, and the mean values (given in degrees of strength) (4) for the four animals plotted against the days when the samples were taken

It is seen that even the first two injections cause a considerable production both of antipolysaccharide and of antilipid antibodies (reagins) These reagins, however, have disappeared two weeks after the last injection, while the sera still have a distinct content of antipolysaccharide antibody 6 weeks after completion of immunization

Contrary to what has been stated elsewhere (5, 6, 7), a distinct production of reagins was observed during immunization in the present study, but these antibodies disappeared with surprising rapidity Therefore, the blood samples, usually taken 5 days or more after the last injection of treponemes, showed little or no content of reagins *Poetschke & Killisch* (12) also noted a production of reagins, when rabbits were immunized with Reiter treponemes These antibodies were demonstrated by the VDRL microflocculation test, while a complement-fixation test was not used

SUMMARY

The origin of the Reiter-treponeme is mentioned together with techniques of cultivation and immunization It is stated that immunization with lyophilized Reiter-treponemes causes a marked production of at least two types of antibodies namely [illegible]
antibodies However, the an
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period

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THE RICKETTSIA AND VIRUS DEPARTMENT STATENS SERUMINSTITUT COPENHAGEN

THE WESTERN BOUNDARY OF ENDEMIC TICK BORNE MENINGO ENCEPHALITIS IN SOUTHERN SCANDINAVIA

Surveys in Denmark

By

F A FREUNDT

Received 28 IX 62

Knowledge concerning the geographical distribution of the Russian spring summer group of tick borne encephalitis viruses (the RSS or RSSE complex) in Eastern, Northern and Central Europe has been rapidly expanding during recent years It has particular bearing on the present study that extensive endemic areas have been demonstrated in the territories surrounding the Baltic Sea the Leningrad district of the USSR (7), Southern Finland (6, 8, 12) and Sweden (5 14, 16–19), Poland (9), and Eastern Germany (13)

Up to the present there are no published reports on the possible occurrence of the RSS viruses in Western Germany In Southern Sweden the frequency of cattle sera containing RSSE antibodies decreases gradually from east to west an extremely low incidence of positives being found in the west coast regions (19)

On the [illegible] fac[illegible] geo[illegible] mig[illegible] to represent a possible exception, located as it is in the western area of the Baltic Sea about 40 km south of Sweden and 100 km north of Poland and Eastern Germany Moreover, observation on Bornholm of apparent causality between tick bites and diseases of the central nervous system (CNS) was reported several years ago (3, 4)

During the years 1958 to 1962 surveys were made of h[illegible] animal [illegible] evidence [illegible] the RSS [illegible] as obtained from Bornholm only, a brief introductory description of this island should be given

The author [illegible] collecting anir[illegible] thanks are du[illegible] County Hospi[illegible] n making available case records and arranging contacts with previous patients and to Dr S F *Koford* County Medical Officer for valuable suggestions and assistance in providing material for examination

REFERENCES

1 *Beck A* The role of the spirochaete in the Wassermann reaction J Hyg 39 298 1939
2 *Brewer J H* Clear liquid mediums for the aerobic cultivation of anaerobes J Amer Med Ass 115 598 1940
3 *Christiansen Aa Hein* Studies on the antigenic structure of *Treponema pallidum* 2 Isolation and purification of polysaccharides from Reiter s apathogenic strain WHO/VDT/RES/15 1962
4 *Christiansen Aa Hein* Studies on the antigenic structure of *Treponema pallidum* 3 Antigenic properties of polysaccharides isolated from Reiter s apathogenic strain WHO/VDT/RES/16 1962
5 *D Alessandro G* Personal communication 1961
6 *Eagle H & Germuth F G* The serologic relationship between five cultured strains of supposed *Treponema pallidum* (Noguchi Kroo Nichols Reiter and Kazan) and two strains of mouth spirochaetes J Immunol 60 223 1948
7 *Faure M & Pillot J* Composition antigénique des tréponemes Ann Inst Pasteur 99 729 1960
8 *Gates F I* Cultivation of anaerobic treponemata J Exper Med 37 311 1923
9 *Gaehtgens W* Theoretisches und Praktisches uber die Wirkung eines karbolisierten wässerigen Pallidaantigens Ztschr Imm forsch 63 398 1929
10 *Klopstock F* Die Entstehung der syphilitischen Blutveränderung und ihr Nachweiss mittels alkoholischen Spirochaeten Extrakts Dtsch med Wschr 52 226 1926
11 *Muller P & Nothaas R* Superinfektionsversuche mit einem durch Verimfung von Kulturspirochaeten (Reiter) in den Kaninchenhoden gewonnenen Stamm Munch med Wschr 1 169 1928
12 *Poetschke G & Killisch L* Untersuchungen mit fluoreszenzmarkierten Antikorpern Schweiz Ztschr all Path Bakt 22 765 1959
13 *Reiter H* An account of the so called Reiter treponeme Brit J vener Dis 36 18 1960
14 *Reiter H* Praktische Verwertung von Reinkulturen von Spirochaete dentium und pallida Derm Wschr 83 1199 1926a
15 *Rose N R & Morton H E* The morphologic variation of treponema Amer J Syph 36 17 1952
16 *Wallace Alwilda L & Harris A* Preparation of Reiter protein antigen Public Health Lab 10 27 1958
17 *Wassermann A v & Ficker M* Reiterkulturen der Spirochaete pallida im festem und flussigem Nährboden sowie Übertragung dieser Kulturen auf Tiere Klin Wschr 1 1101 1922
18 *WHO/VDT/RES 8* Treponema pallidum 1961

a formalinized mouse brain suspension followed by repeated and increasing doses of live tissue culture virus (mixed chick embryo cells) No overt effect was observed in the animals

Preparation of sera for tests —All sera were inactivated at 56° for 30 minutes and stored at —20° C

Nonspecific serum inhibitors of arbor virus agglutinins were removed for the HI test as prescribed by *Clarke & Casals* (2) by adsorption to kaolin With some sera however the standard procedure using a 25 per cent suspension of kaolin was not sufficient for the complete removal of non specific inhibitor and therefore either adsorption with higher concentrations of kaolin or preferably acetone ex

centrated rehydrated antigen was stored in the wet frozen state in sealed ampoules at —60° C

Technique of the HI test —Clarke & Casals technique was used with a few minor modifications The thawed antigen diluted in an adequate volume of bovine albumin borate saline to give an estimated titer of 4 8 units per 0 2 ml was usually kept at +4° C for at least two or three days before use instead of one hour as recommended by Clarke & Casals This was done because the titer constantly increased during the first few days after dissolving after which period a fairly stable level was maintained for a considerable length of time The time required for the antigen to reach its maximum titer increased the higher the degree of dilution

The HI test was carried out in lucite plates with twofold dilutions of sera in borate saline with pH 9 0 After overnight incubation at +4° C of the serum antigen mixtures equal volumes were added of a 0 33 per cent suspension of washed goose erythrocytes in the diluent adjusted to give the final pH of 6 4 f und to be optimal for the RSS antigens Reading of the test was made after the red cells were allowed to settle for one hour at 37° C. In addition to the serum control without antigen added which served to reveal any possible non specific haemagglutination the inclusion of the inhibitor control introduced by *Salminen* (11) and *Salminen et al* (12) proved very useful This test is based on the fact that the non specific inhibitory effect of sera which is apparently due to certain lipid complexes is heat resistant and can withstand boiling for at least one minute while the antibodies

l ace vernight at +4 C and the test proper was carried out at 37° C for one hour The titer was read as the highest dilution giving at least 50 per cent inhibition of haemolysis

Technique of the neutralization test —The virus neutralization test was performed with ten fold serial dilutions of virus against undiluted inactivated serum 10 per cent suspensions of infected mouse brain stored at —60° C were diluted in PBS to which 10 per cent inactivated rabbit serum had been added The serum

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LD_{50} Positive sera were subsequently titrated to the endpoint and the LD_{50} and logarithmic neutralization index calculated according to the method of *Reed & Muench*

RESULTS

1 *Surveys of Human and Animal Sera*

A summary of the overall results obtained with the HI and neutralization tests are shown in *Table 1* With a few exceptions indicated in

The size of Bornholm is 587 square kilometres Although geologically a rocky island 65 per cent of its area is cultivated land while 17 per cent (100 km²) is covered by forest This is partly mixed coniferous and leaf forest with a dense underbrush Roe deer were imported into the island at the end of the 19th century and are now very numerous Cattle keeping is extensive but there are few sheep and goats Bloodsucking ticks (*Ixodes ricinus*) are prevalent as they are in several other forest districts in Denmark The population of Bornholm is about 50 000 but in addition the island is a very popular holiday resort

MATERIALS AND METHODS

Collection of blood samples—Blood samples from deer and red deer were collected during the hunting season in the late autumn and early winter Usually, the blood was taken from the exposed chest cavity a few hours after the deer had been shot Occasionally, additional blood samples were obtained by cutting the veins of the neck immediately after the animals were killed since this method yielded less contaminated specimens It should be mentioned here however that although some samples collected from the chest cavity were more or less decomposed by bacterial growth on arrival at the laboratory the serological results with these specimens and with those obtained by venesection were essentially identical

Blood was procured from cows by puncture of the superficial jugular vein In the case of birds small amounts of blood could usually be withdrawn from the chest cavity and the heart

Generally all specimens were received in the laboratory within 24 hours after collection

Collection of ticks and attempted virus isolation—Ticks were collected by dragging white flannel rags fixed to the end of sticks throug the grass and underbrush Particularly high yields were obtained from lairs in the forest floor vacated recently by deer The ticks were transported to the laboratory in glass vials or tubes containing moist grass and moss and covered with filter paper Pools of live ticks (about 100 larvae 50 nymphs or 25 imagoes) were ground in mortars with sand and 1 2 ml of diluent consisting of equal parts of phosphate buffered saline and inactivated rabbit serum The supernate obtained from centrifugation in the cold (2500 r p m for 10 minutes) was used for intracerebral inoculation into suckling and adult mice Three subsequent blind passages of 10 per cent brain suspensions were carried out at intervals of 8 10 days In a few additional experiments batches of about 30 adult ticks *were allowed to feed on suckling mice (in groups of six) for about four* to ten hours during which the baby mice were withdrawn from their mothers After observation periods of 8 to 10 days blind passages of brain material were carried out in the usual manner

Attempts were made to isolate virus from birds by intracerebral passages of 10 per cent suspensions of spleen liver kidneys and brain into mice During transportation of the birds or their organs to the laboratory a low temperature was maintained by means of CO_2 ice

Test viruses—Two laboratory strains of the RSS complex were employed as test viruses in the serological surveys a Louping ill strain (LI) and the Czech B3 strain of Central European encephalitis kindly provided by *Dr A Svedmyr* Stockholm and *Dr F Benda* Prague The strains were further propagated in this laboratory by intracerebral passages in mice and stored as 10 per cent brain suspensions in sealed ampoules in a CO_2 ice cabinet *The diluent was phosphate buffered saline* (PSB) containing 10 per cent inactivated rabbit serum

Except when otherwise stated the B3 strain was employed throughout the present study for the haemagglutination inhibition (HI) and complement fixation (CF) tests The neutralization test was carried out at the beginning of the survey with the LI strain while subsequently this was replaced by B3 as the seed virus

Reference serum—A lyophilized immune diagnostic horse serum prepared against the Czech HYPR strain by the Institute of Virology of the Czechoslovak Academy of Sciences in Bratislava and supplied by *Prof D Blaskovic* was used as reference serum According to the declaration both infectious tissue culture fluid (chicken fibroblasts) and mouse brain suspension had been used for the immunisation procedure

Preparation of hyperimmune sera Sheep and guinea pig hyperimmune sera against the *LI strain* were *prepared* by the subcutaneous injection of one dose of

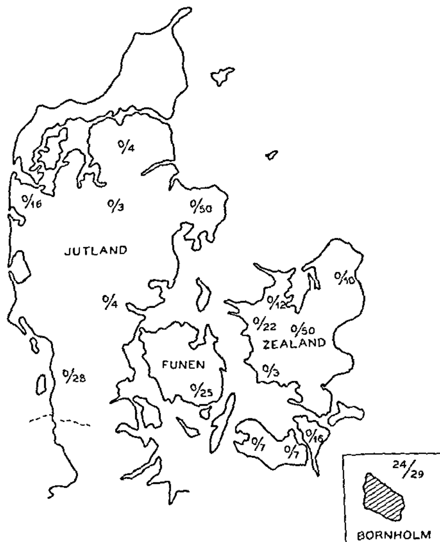


Fig 1
Geographical distribution in Denmark of deer sera tested for RSSE antibodies
Numerator number of positive specimens
Denominator total number of specimens tested from each district

On the other hand, no positive sera were found among 134 specimens collected from cattle from other parts of the country during 1958–59

Birds — A limited number of sera were obtained from birds on Bornholm Rooks (*Corvus frugilegus*) were selected for examination, because these are said to be particularly liable to infestation with ticks, which sometimes even form a heavy collar round the neck of the birds The popular word used on Bornholm for ticks, "rook's itch", refers to

the table, the results achieved with these two tests were in complete agreement throughout the survey. On the other hand, the CF test is not included in the table, because it was either less frequently positive or else it could not be used due to a pronounced anticomplementary effect.

It will be seen from the table that the positive sera, whether from man or animals, are confined exclusively to Bornholm. In the following more detailed description of the individual groups, reference should be made throughout to this table.

TABLE 1

HI and Neutralization Tests with RSSF Viruses

Sera tested	Bornholm			Other parts of Denmark	
	No sera	No positive	Per cent pos	No sera	No positive
Deer	29	24 (+3)*	83	257	0
Cattle	135	4 (+2)*	3	134	0
Rooks	8	0			
Forest workers	40	12	30		
Cases of meningo encephalitis etc	12	8	(67)	545	0
Other population groups	508	7	1.4	305	0

* Sera positive in HI or neutralization tests only

Animal Sera

Deer.—Sera from deer (*Capreolus capreolus*), and, to a lesser extent, from red deer (*Cervus elaphus*) were chosen as principal screening material, because these animals are known to be heavily infested with ticks from early spring to mid-autumn.

Among 29 samples of deer sera from Bornholm, both HI and neutralizing antibodies were demonstrated in no less than 24 cases, *i.e.* an incidence of 83 per cent. A further three sera were positive in one test only. The individual titers are shown in *Table 2*. Among 12 sera that were not anticomplementary, CF antibody was found in one sample only (No. B 3), to a titer of 8.

In striking contrast, all the 257 sera from deer (including 10 red deer) collected from other parts of Denmark (as shown in the map *Fig. 1*) were completely negative.

Cattle.—The incidence of positive reactors in cattle from Bornholm was much lower than among the deer. Out of a total of 135 sera both HI and neutralizing antibodies were demonstrated in four, and neutralizing antibody in a further two cases only. It should be mentioned that two of the positive sera were obtained from a particularly selected group of 19 cows representing two herds which were known definitely to be seasonally infested with ticks. Moreover, the tick borne protozoan disease, *Piroplasmosis* or *Babesia*, had previously been observed in cattle grazing in that particular district of Bornholm (15).

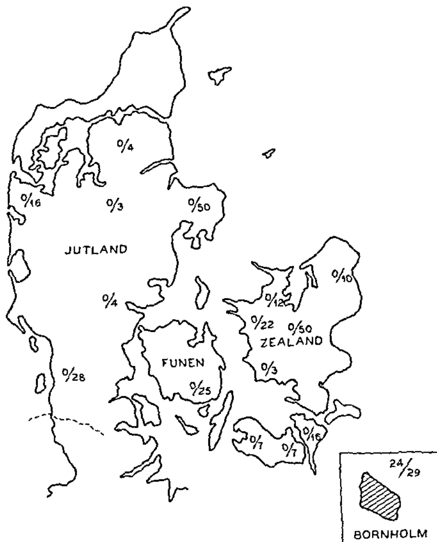


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this observation However, only one tick was found in one of the eight young rooks examined, and HI antibodies could not be demonstrated in any of the sera The amounts of serum were too small to permit the performance of other tests

TABLE 2

Deer Sera from Bornholm
HI and Neutralizing Antibody Titers against Czech B3 Virus

Serum no	HI	Log NT	Serum no	HI	Log NT
B 1	20	2 9	B 17	80	4 0
B 2	80	3 1	B 18	20	0 9
B 3	80	2 3	B 19	80	2 2
B 4	80	2 1	B 20	10	neg
B 5	40	3 3	B 21	20	3 3
B 6	20	3 3	B 22	320	3 9
B 7	80	3 7	B 23	20	3 3
B 8	40	3 3	B 24	20	neg
B 10	< 10	2 7	B 25	80	2 7
B 12	160	3 9	B 26	40	3 9
B 13	80	3 0	B 27	80	2 2
B 14	40	3 8	B 28	20	2 9
B 15	160	2 2	B 29	80	3 5
B 16	40	2 5			

Sera collected November 1960

Human Sera

Cases of infectious disease of the central nervous system (CNS) —In the course of the present investigations no acute cases of tick-borne meningo-encephalitis in Bornholm happened to come to our attention However, of the 79 patients admitted to the County Hospital of Bornholm with a diagnosis of acute meningo encephalitis during the ten-year period 1951 60, 12 (including the three forest workers mentioned below) were selected for a retrospective serological study carried out in December 1960 As will be seen from *Table 5*, HI and neutralizing antibodies against RSSE virus were present in fairly high titers in eight of these persons, while CF antibody persisted in five cases

A total of 717 sera were obtained from the rest of the country during the period June 1958 to early May 1962, from 545 patients with tentative diagnoses of acute, subacute or chronic meningo encephalitis, serous meningitis, poliomyelitis, polyradiculitis, and allied conditions Paired sera were provided in about one-third of the cases, and nearly half of the remaining cases from which only one specimen was available were definitely known to have lasted for at least 14 days This material originated from all parts of the country, although the majority of the sera were obtained from the *Department of Communicable Diseases*, Blegdamshospitalet, Copenhagen, and from the *Department of Neuro-*

logy Municipal Hospital Arhus The latter receives patients from most of Jutland Not one of these sera contained RSSE antibody

Forest workers—Sera were collected in December 1960 from 40 forest workers and other persons who on account of their profession were particularly exposed to tick bites in the forests of Bornholm the majority for several years Serological evidence of previous contact with RSSE virus was found in 12 (30 per cent) of these persons (*Table 3*) all of whom had both neutralizing and HI antibodies The CF test was positive less frequently although in one person a titer of 128 was demonstrated The latter was still convalescent from a case of acute meningo encephalitis the onset of which had occurred less than six months earlier Two other positive forest workers had been admitted to hospital with a diagnosis of acute meningo encephalitis three and seven years previously These cases will be described in more detail together with other cases listed in *Table 5*

TABLE 3

Forest Workers etc from Bornholm
HI CF and Neutralizing Antibody Titers against Czech B3 Virus

Serum no	Age	HI	CF	Log NI
Sk 8	57	40	0	2 1
Sk 9	41	40	0	2 4
Sk 10	35	80	0	3 3
Sk 20	36	160	8	2 1
Sk 24	54	320	16	3 6
Sk 25	53	160	4	3 1
Sk 28	51	80	0	4 1
Sk 29	62	40	0	2 5
Sk 32	55	80	2	3 0
Sk 35	60	160	2	2 7
Sk 38	47	160	0	2 5
E 411	19	160	128	3 2

Sera collected December 1960

Blood samples from forest workers were not collected from other parts of the country because the results obtained with deer sera did not encourage the rather difficult collection of such material

Other population groups—508 residents of Bornholm from whom blood samples were sent to the Statens Seruminstitut in September November 1961 for the performance of routine serological tests (Wassermann etc) were chosen to represent at 1 per cent random sample of the total population of the island This group consisted of 202 males and 306 females and the majority ie 70 per cent were healthy individuals either blood donors or pregnant women Th... ung were ...ges ran... 20 to 50

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the hospital records of six patients and the remaining two persons definitely claimed when interrogated in 1960 that they remembered having been bitten by ticks prior to their illness. The interval between exposure and onset of disease ranged from a few days up to two weeks.

TABLE 5

Retrospect Serological Survey of Cases of Meningo Encephalitis in Bornholm

Serum no	Age	Sex	Onset of disease	Tick bites	Biphasic course	Spinal fluid cell counts	Antibody titers 1960		
							HI	CF	log NI
Sk 28	43	male	1953 July	+	?	631/3	80	0	4.1
F 403	36	female	1957 July	+	—	76/3	2560	2	4.0
F 407	48	female	1957 July	+	+	296/3	640	8	3.2
E 408	56	male	1957 August	+	+	39/3	320	0	3.4
F 410	39	male	1957 August	+	+	208/3	320	4	3.0
Sk 9	38	male	1957 August	+	?	12/3	40	0	2.4
F 404	43	male	1958 August	+	?	75/3	160	8	3.4
F 411	19	male	1960 July	+	+	518/3	160	128	3.2

Sera collected December 1960.
Age of patients refers to the year of disease.

At least four cases had followed a typical biphasic course: an initial feverish influenza-like phase of about one week's duration, succeeded by a relatively symptom-free interval of two or three days before the occurrence of a secondary high rise of temperature accompanied by symptoms from the CNS. In the apparently monophasic cases these symptoms prevailed from the beginning. They were characterized by severe and persistent headache, periorbital and retrobulbar tenderness, vomiting, vertigo, rigidity of the neck and back, Kernig's sign, myalgia and (in one case) insomnia. Bilateral Babinski's sign was observed in one patient, and paresthesia of the fingers of the right hand followed by the development of transient paralysis of the arm and shoulder region occurred in another case.

The spinal fluid showed a slight to moderate increase of predominantly mononuclear white cells and increased content of protein. The erythrocyte sedimentation rate ranged from 8 to 40 mm per hour on admission to hospital.

In addition to the cases recorded in Table 5, one of the persons included in the survey of the general population of Bornholm (*Table 4, No. 318*), the young girl mentioned above, probably suffered from the sequelae of a mild attack of meningo-encephalitis since high titers of antibodies against RSSF virus were detected accidentally in a blood sample submitted for other purposes. The supposition that the antibody found in this patient was a response to a recent infection was further substantiated by the demonstration of a subsequent rapid decline of the HI antibody. Although no examination of the spinal fluid was made

TABLE 4

Non-Selected Residents of Bornholm

Incidence and Titers of HI, CF and Neutralizing Antibody against Czech B3 Virus

Total no of persons tested				Positive reactors				
Sex	No sera	No positive	Per cent positive	Serum no	Age	Antibody titers HI	CF	log NI
Females	306	3	1 0	48	48	80	4	3 4
				318	18	1280	64	2 9
				342	50	320	8	3 9
Males	202	4	2 0	5	29	320	8	4 2
				127	50	640	8	3 9
				275	45	160	8	≥4 2
				388	76	80		3 1
Totals	508	7	1 4					

Sera collected September November 1961

The results of the serological survey are presented in *Table 4* A total of seven positive reactors were found, *i e* 1 4 per cent, with a distribution according to sex of 1 per cent in females against 2 per cent in males In addition to high titers of HI and neutralizing antibodies, CF antibody was demonstrated in all except one case which was not examined by this test Particularly high titers were found in the serum (No 318) of an 18-year-old female who had been admitted to hospital with symptoms of an infectious disease Two subsequent blood samples from this patient obtained two and six weeks later showed no change of the antibody level with the CF and neutralization tests, while the HI titer decreased from 1280 through 320 to 160 in tests carried out simultaneously with all three specimens Except for this patient (see below), no information was provided by the sero-positive persons in this group concerning previous disease resembling clinically infections caused by RSSE virus

For comparison, 305 sera were collected in April May (Nos 1–102), and in August (Nos 103–305), 1961, from blood donors in Copenhagen, who according to specific inquiry had never been to Bornholm All of these were completely negative

2 *Clinical Picture of Cases of Meningo-Encephalitis on Bornholm*

In *Table 5* a brief summary is given of the available clinical data concerning the eight cases of meningo-encephalitis where RSSE antibodies were demonstrated by a retrospective survey carried out from a few months and up to seven years after the infection

All of these cases had occurred in the months of July and August Mention of tick-bites shortly before the onset of disease was found in

The results of comparative CF titrations of both human sera and LI hyperimmune sera from sheep and guinea-pig against the Czech B3 and the English LI strains are shown in *Table 6* (The Czech horse hyperimmune serum was unsuitable for the CF test) The figures in the table show a definite tendency throughout the experiment towards higher titers in the human sera when tested against the B3 antigen One particular serum (No E 411) shows a 16-fold difference, confirmed in repeated experiments, in the reactivity against the two antigens The fact that both of the two LI hyperimmune sera yielded lower titers with the B3 than with the homologous antigen would seem to exclude the possibility of a slightly different adjustment of the experimental conditions as a likely explanation of the diverging results obtained in the parallel tests

Similar results were obtained in experiments performed with the HI test, using sera from deer against four units of each antigen (*Table 7*) As a general rule, significantly higher titers were found with the Czech strain In cross tests between the two different antigens and their corresponding hyperimmune sera, the homologous systems provided the higher titers

In comparative neutralization tests carried out with a limited number of field sera, neither of the two type strains was consistently neutralized to a higher titer than the other As a matter of fact, the neutralization indices obtained against the B3 strain differed from those found with the LI strain within the fairly narrow range of 0 1 to 0 5 logs only On the other hand, with the exception of one case (perhaps due to experimental error) in repeated experiments the hyperimmune sera neutralized their homologous strains to significantly higher titers than the heterologous, with a difference of 0 6 to 2 1 logs (*Table 8*)

TABLE 7

Comparative HI Titrations with Deer and Hyperimmune Sera against 4 Units of Czech B3 and LI Antigens

Serum no	HI titers		Serum no	HI titers	
	B3	LI		B3	LI
B 1	20	<10	B 19	80	20
B 5	40	10	B 20	10	<10
B 6	20	<10	B 21	20	<10
B 7	80	20	B 22	320	40
B 8	40	<10	B 23	20	10
B 12	160	80	B 24	20	10
B 13	80	80	B 26	40	10
B 14	40	<10	B 27	80	10
B 15	160	40	B 29	80	10
B 16	40	<10			
B 17	80	20	LI F46*	40	80
B 18	20	<10	HYPR,H§	1280	320

* LI sheep hyperimmune serum
§ Czech HYPR horse hyperimmune serum

and no neurological signs were noted on clinical examination her symptoms on admission to hospital—fever of unknown origin together with severe headache—would seem to be compatible with the diagnosis suggested. The case is mentioned here to illustrate a clinical type of RSSE infection that is liable to escape aetiologic diagnosis.

3 *Attempted Virus Isolation*

Acute phase material from patients suitable for virus isolation (blood and spinal fluid) were not available during the course of the present investigations.

A total of 630 hungry ticks (*Ixodes ricinus*), including 113 larvae, 353 nymphs, and 164 imagoes (79 males and 85 females) were collected at various periods during the summer and early autumn of 1961 in the forests of Bornholm. However no virus could be isolated from the ticks either by conventional mouse brain passages of ground material or by allowing live ticks to feed on suckling mice.

Since it is suggested in the literature (*10*) that birds may serve as chronic reservoirs for arbor viruses, attempts were also made to isolate RSSE virus from the spleen, liver, kidneys and brain of 12 rooks (*Corvus frugilegus*) and one jackdaw (*Coleus monedula*). These experiments were also negative.

4 *Comparative Titration of Positive Sera against the B3 and TT Strains*

Since isolation of virus from ticks was entirely unsuccessful, any approach to defining the relationship of the responsible virus on Bornholm to known sub-groups within the RSS complex must be based on cross tests between type strains of the RSSE/TT viruses and positive field sera.

TABLE 6

Comparative CF Titrations with Human and Hyperimmune Sera against 2 Units of Each B3 and TT Antigens

Serum No.	CF titres B3	CF titres TT
F 403	2	0
F 404	8	2
F 407	8	8
F 410	4	0
F 411	128	8
Sk 20	8	0
Sk 24	16	8
Sk 25	4	0
Sk 32	2	0
Sk 33	2	0
Sk 35	2	0
LI F 45‡	16	32
TT/Ma 11§	32	64

‡ TT sheep hyperimmune serum
§ TT guinea pig hyperimmune serum

of persisting maternal antibody can probably be ruled out at that age, this observation suggests a fairly massive exposure to infected ticks in early life

To what extent, then, does RSSE virus cause clinical disease in man in Bornholm?

As mentioned in the introduction, the observation of an apparent causality between tick-bites and disease of the CNS in Bornholm was made many years ago The possible aetiological implication of tick-borne encephalitis virus was even considered in two cases of polyneuritis and encephalitis published by *Dalsgaard Nielsen* (3), and *Dalsgaard-Nielsen & Kierkegaard* (4), who, however, concluded that an allergic response to the tick salivary secretions was a more likely explanation for the CNS symptoms As a matter of fact, certain peculiar clinical features such a the development in one of the cases (4) of a widespread migratory chronic erythema from the site of the tick-bite, might suggest the involvement of allergy or hypersensitivity in the pathogenesis of these particular cases

On the other hand, there is little doubt that the eight cases of meningo-encephalitis described in the present study were actually caused by RSSE virus Although the serological diagnosis is entirely based on the results of a retrospective survey which did not allow the examination of paired acute phase and convalescent sera, there is at least complete agreement between the presence of antibodies, the history of tick-bites, and the clinical and laboratory findings, as well as the course of the disease The general clinical picture of the Bornholm cases of meningo-encephalitis as a relatively mild disease with a low frequency of severe neurologic symptoms followed by complete recovery with no sequelae, is in fair accordance with the clinical data reported from Sweden and Finland

Undoubtedly, a number of cases of RSS infections in man either do not develop symptoms from the CNS at all, or else are not sufficiently distinct to be recognized as such, as illustrated by one case in the present material

Naturally the ratio of clinical cases of meningo encephalitis to cases showing influenza like symptoms only, or to inapparent infections, can not be determined, on the basis of the available material from Bornholm However, the occurrence of no more than three recognized cases of meningo-encephalitis among 12 sero-positive forest workers suggests that the incidence of more serious disease is relatively low

The total morbidity among the population of Bornholm, in terms of clinically manifest cases of tick-borne meningo encephalitis, is also obscure although a rough estimate of maximum figures can be deduced from the information available concerning the total number of cases of meningo-encephalitis found in Bornholm

During the 10 year period 1951–60, a total of 79 cases of acute meningo encephalitis, including 62 males and 17 females, were admitted to

TABLE 8

Comparative Neutralization Tests with Human Deer and Hyperimmune Sera against Czech B3 and LI Viruses

Serum no	Log NI	
	B3	LI
L 404	2 9	3 4
F 411	3 5	3 2
Sk 8	2 2	2 1
Sk 9	2 6	2 4
B 12	2 9	3 1
B 14	2 8	3 3
B 15	3 2	2 7
LI/I 45*	2 8	4 2
"	2 2	4 3
"	2 2	≦ 3 7
HYPR/HS§	5 0	4 4
"	4 0	4 0
"	4 8	3 8

* LI sheep hyperimmune serum
§ Czech HYPR horse hyperimmune serum

DISCUSSION AND CONCLUSIONS

The aim of the present study was to determine whether any activity of encephalitis virus of the RSS-complex could be demonstrated in Denmark as neighbour to the eastern endemic area

Although no virus isolations have been achieved up to the present, the demonstration of HI, CF and neutralizing antibody in a number of human and animal sera from Bornholm provides convincing evidence that this island represents an endemic focus of infection caused by tick-borne encephalitis virus

The antibody rate of 1 4 per cent found in the general population of Bornholm is about the same level as the one recently demonstrated in, for example, south-eastern areas of Finland (6), though notably lower than the one reported for other regions such as the Finnish Aland archipelago where the average rate is 13 per cent (12)

Among the animals examined, the incidence of positive reactors found in cattle (3 per cent) was close to that found in the human population

However, neither the low antibody rates shown by these two groups of sera nor the failure to isolate virus from ticks and other sources, would appear to provide a true picture of the full extent of the RSSE virus activity on Bornholm Actually, the extremely frequent occurrence of antibody *in particularly exposed groups of humans and animals*, such as forest workers (in 30 per cent) and deer (in 83 per cent), clearly shows that tick-borne encephalitis virus is indeed very prevalent in Bornholm, thus providing a not inconsiderable potential risk of infection In this connection it might be mentioned that there were at least five six-months-old kids *among the positive deer Since the possibility*

also observed, though to a less marked extent, in different districts of Southern Sweden (19) Although in some places cattle graze near to trees and bushes, for instance in small enclaves bordering on forests, the majority of the cattle in Bornholm graze in open arable fields that are only occasionally infested with ticks The finding of two positive cows out of 19 (i e 10 per cent) from selected herds known to be seasonally infested with ticks provides some evidence in support of this explanation

From these observations the conclusion can be drawn that the value of cattle sera as basis for study of the geographical distribution of tick-borne viruses is highly dependent on local conditions While very useful for instance in extensive areas of Sweden and Finland, serological surveys based solely on material collected from cattle might be very misleading in typical agricultural countries like Denmark

To the knowledge of the author, very few detailed reports have been published regarding the study of deer sera for RSSE antibodies In Czechoslovakia, CF antibody was demonstrated in one, and neutralizing antibody in four, out of 16 sera from deer and related species (1) Negative results were reported from East Germany, but the number of sera is not mentioned (13) However, as shown by the Bornholm survey, sera from deer would, like sera from other wild animals more or less constantly confined to woodland, appear to reflect quite sensitively in any conditions the possible endemic occurrence of tick-borne virus infections

Therefore, when discussing the significance of the entirely negative results obtained with a variety of sera from by far the greatest part of Denmark, particular emphasis should be placed on the absence of RSSE antibody in a total of 257 deer sera representing most of the larger forest areas of the country (cf *Fig 1*) It should be added that, on the whole, *Ixodes ricinus* is just as prevalent in these areas as in Bornholm

It can finally be conluded that at present the whole of Denmark, except Bornholm, is outside the eastern endemic area of Central Euro- [illegible]

[illegible] show a pronounced decrease of the rate of RSSE antibody towards the western coastal regions of Southern Sweden

Future investigations in Denmark might contribute to solving the interesting and important problem as to whether or not the endemic zone is gradually expanding

SUMMARY

Using haemagglutination inhibition, complement fixation, and neutralization tests, a study of the occurrence of antibodies against the

the County Hospital of Bornholm The annual number of cases varied from 1 to 18 On the basis of a cautious evaluation of seasonal occurrence, clinical course, spinal fluid findings, etc, a maximum number of 39 cases (including the eight sero-positive persons) may have been caused by RSSE viruses This figure would not seem to need any significant correction due to the possible erroneous diagnosis of some cases of tick-borne meningo-encephalitis as polio, since apart from a total of 133 cases of polio during the big epidemic in 1952-53, only two additional cases were recorded in the period 1951-60

The conclusion is that tick-borne meningo-encephalitis in Bornholm would not appear to represent a serious problem at present, either from epidemiological or clinical points of view

The endemic focus of RSSE encephalitis thus demonstrated in Bornholm should be seen, of course, in its proper perspective as part of the vast endemic area surrounding the Baltic Sea In this connection, it is of interest to note that judging from the results of the CF and HI tests with field sera, the virus prevalent in Bornholm is more closely related to the Central European group of viruses than to the LI virus This is in accordance with the results obtained by *von Zeipel & Svedmyr* (17), who in box titrations with a pool of Swedish convalescent sera against antigens prepared from the LI and Czech HYPR strains found four to eight times higher serum titers with the latter These authors also found a tendency to slightly higher titers against the HYPR strain in cross neutralization tests using the serum dilution technique Their results were further substantiated by the antigenic analysis of Swedish virus strains subsequently isolated by *von Zeipel* (16)

The fact that Bornholm has been proved to belong to the well-known endemic area of encephalitis in Central Europe is only one aspect of the present study and perhaps the least interesting since, on the whole its adds very little to our knowledge Greater importance should perhaps be attached to the fact that not one single positive result was obtained by examination of a fairly large number of animal and human sera, including many samples from cases of acute meningo-encephalitis, from other parts of Denmark

However, in order to evaluate properly these negative results, it will be of interest to consider the validity of sera from different sources as screening material Though unnecessary to point out the obvious significance of sera from cases of meningo-encephalitis, a few comments should be made on the use of cattle and deer sera as indicators of a possible activity in nature of tick-borne viruses

Actually, the infrequent general occurrence of RSSE antibodies in cattle from Bornholm is remarkable compared with the high frequencies found in south-eastern regions of Sweden (19), and in some areas of Finland (6), Poland (9), and Czechoslovakia (1) The explanation for this apparent discrepancy should probably be sought in the influence of different grazing conditions on the exposure to tick-bites

8 *Oker Blom N* Kumlinge disease Ann Med Exper Fenn 34 309 1956
9 *Przesmycki F Taytsch Z Wroblewska Z & Lachmayer J* Investigation of a natural focus of encephalitis in the Puszcza Bialowieska national park J infect Dis 106 276 1960
10 *Reeves W C* Overwintering of arthropod borne viruses Progr med Virol 3 59 1961
11 *Salminen A* Haemagglutination with tick borne encephalitis viruses Acta virol (English ed) 4 17 1960
12 *Salminen A Eriksson A W & Oker Blom N* Haemagglutination inhibiting ick

13 12

14 *Svedmyr A von Zeipel G Holmgren B & Lindahl J* Tick borne meningo encephalomyelitis in Sweden Arch ges Virusforsch., 8 565 1958
15 *Lind R* Personal communication 1960
16 von *Zeipel G* Isolation of viruses of the Russian spring summer encephalitis louping ill group from Swedish ticks and from a human case of meningo encephalitis Arch ges Virusf rsch 9 460 1959
17 *von Zeipel G & Svedmyr A* Growth of viruses of the Russian spring summer usf rsch 8 370 1958
18 *von* Tick borne meningo
19 *von* raphical distribution in Sweden of viruses belonging to the Russian spring summer louping ill group Arch ges Virusforsch 9 449 1959

RSS complex of encephalitis viruses was carried out on sera from animals and man collected in different areas of Denmark

Evidence of RSSE virus activity was obtained from one area only, *viz* Bornholm

A high incidence of positive reactors was found in forest workers (30 per cent) and in deer (83 per cent), *i e* in selected groups particularly exposed to tick-bites

The antibody rate in the general population was 1–2 per cent

In a retrospective study of sera from a limited number of persons who had been admitted to hospital in recent years with a diagnosis of acute meningo-encephalitis, antibodies were demonstrated in eight cases, all of which had a history of tick-bites shortly before the onset of the disease

The results of comparative HI and CF titration of positive sera against a Louping ill and the Czech B3 strain suggested a closer relationship between the responsible virus in Bornholm and the Central European, rather than the Louping ill group of viruses

Attempts to isolate virus from ticks and birds were unsuccessful

The significance of the negative results obtained with a great number of sera from other parts of Denmark is discussed

ADDENDUM

A preliminary survey of the possible occurrence of HI antibody against Group A arbor viruses, using a *Sindbis* strain as test virus, was made with 357 sera *from* cases of meningo-encephalitis, etc *from the* whole of Denmark In addition, positive sera from Bornholm were also tested with the Sindbis antigen (at pH 6 2) in order to provide further proof of the specificity of the haemagglutination inhibition against RSS virus found with these sera However, the results were completely negative

REFERENCES

1 *Blaskovic D* (Editor) Epidemia Encefalitidy v Rožňavskom *Prirodnom Ohnisku* Nákaz Vydavatelstvo Slovenskej Akadémie Vied Bratislava 1954 (in Czech with an English summary)

2 *Clarke D H & Casals J* Techniques for haemagglutination and haemagglutination inhibition with arthropod borne viruses Amer J trop Med Hyg *7* 561 1958

3 *Dalsgaard Nielsen T* Polyradiculitis efter skovflåtbid Nord Med *35* 1754 1947

4 *Dalsgaard Nielsen T & Kierkegaard A* Allergic meningitis and chronic erythma migrans Afzelii after bite by Ixodes reduvius Acta allergol *1* 388 1948

5 [illegible] Tick borne meningo en[illegible] *164* 507 1959

6 [illegible] ical distribution of di[illegible] Exper Fenn *39* 316 1961

7 *Kuznetsova R I Sukhomlinova O I & Churilova A A* The character of biphasic *meningo* encephalitis in the Leningrad oblast J Microbiol Epidemiol & Imm Biol *31* 262 1960

trated toxoids, polyvalent vaccines) The medium was distributed aseptically immediately before use into tubes (14 × 100 mm) with 5 ml in each, after which cotton plugs were inserted This is subsequently referred to as the test medium

Common filtered broth was used for propagation and dilution of the cultures

Inoculum —24 hour broth cultures incubated at the appropriate temperatures, were diluted so as to give a final concentration of a few thousand organisms per ml when 0 1 ml of the dilution was added to 5 ml of the test medium

Incubation —The Pseudomonas strains were incubated at room temperature (about 20° C) and the other strains at 37° C

Observation for growth —The experimental tubes were observed daily for macroscopic growth and when possible counting of viable organisms by plating was ca-

tube was diluted in 15 ml of

m was then poured into a sterile

pe was placed at the appropriate

together with 50 γ per ml (= 1 1 × 10⁻⁴ M) and 100 γ per ml (= 2 2 × 10⁻⁴ M), were chosen for the experiments In our products thiomerosal is used in a concentration of 100 γ per ml (= 2 5 × 10⁻⁴ M) and this concentration together with 25 γ per ml (= 6 2 × 10⁻⁵ M) was investigated where the experimental conditions permitted (see below)

Evaluation of the methods used —It was essential to establish whether the residual concentrations of the preservatives in the broth agar in the plate counting

iable organisms trans-

fter plating is 1-in 150

γ per ml, respectively

ins most sensitive to

and staph albus was not inhibited by 1 γ per ml when grown on broth agar which means that the number of colonies on the counting plates actually expresses the number of viable organisms transferred from the test medium Thus any reduction as compared to the initial number of bacteria in this medium reflects a bactericidal action of phemerol

Similar preliminary experiments with the

broth agar strains like

viable organisms in the test

gly

we

ox

activity of thiomerosal was obtained by diluting it serially in the test medium inoculating the tubes with a few thousand bacteria and incubating them at the appropriate growth temperatures After

material from

RESULTS

Phemerol The antibacterial effect of phemerol on the strains selected for these experiments can be seen from Table 1 In a concentration of

FROM THE SERUM AND TOXOID DEPARTMENT, STATENS SERUMINSTITUT COPENHAGEN

EVALUATION OF THE ANTIBACTERIAL EFFECT OF PRESERVATIVES, WITH SPECIAL REFERENCE TO PHEMEROL AND THIOMEROSAL

By

POVL ELO CHRISTENSEN

Received 28.ix.62

Various procedures have been devised for evaluation of the activity of different antibacterial substances (cfr. the survey of *Reddish* 1957). However, most often the experimental conditions deviate greatly from those prevailing in the environment where the action has to take place. Generally the substances are investigated in media which are optimal for the growth of the test organisms, and with inocula far exceeding those that can be expected to contaminate biological or pharmaceutical products under what are intended to be aseptical conditions of preparation. The selection of test organisms also often seems to be inadequate in relation to conditions of use.

Therefore, the results obtained can only give limited information about the practical value of an antibacterial substance—a fact that has been repeatedly emphasized in the literature on this subject (cfr. *Reddish* 1957).

Since information was required concerning the antibacterial activity of the quaternary ammonium compound, Phemerol® (*Benzetonium chloride*), when used as a preservative for a special vaccine, an experimental technique was adopted which conformed better to the conditions under which the substance was intended to act than the procedures generally recommended.

For comparison, another preservative, thiomerosal (Merthiolate®), with better known antibacterial qualities, was included in the experiments.

MATERIALS AND METHODS

Test cultures.—The following bacterial strains were used to study the antibacterial activity: E. coli, B. anitratum, Alc. faecalis, Proteus vulgaris, Ps. aeruginosa (11 strains), Ps. diminuta, Ps. maltophilia, Ps. fluorescens, Klebsiella, Shigella sonnei, Salmonella paratyphi B, Staphylococcus albus, Staphylococcus aureus (10 strains), B. cereus and B. subtilis. These strains have either been isolated during sterility testing of products from this Institute, are traditionally included in experiments of this type, or might be expected to present special problems.

Media.—Physiological saline containing 1 per cent of horse serum was used for the evaluation of the antibacterial effect. This medium is rather similar to the biological products for which the preservatives were intended to be used (concen-

whether this resistance is specific for strain No 330 or whether it is characteristic for the Pseudomonas aeruginosa group as a whole

For this purpose, ten cultures of Pseudomonas aeruginosa selected at random were incubated at 20° C in the test medium containing 25 and 100 γ of phemerol per ml The results can be seen in Table 2 In a concentration of 25 γ per ml, phemerol inhibited to some extent all ten cultures, but was not able to destroy any of them completely With 100 γ of phemerol per ml eight strains showed no organisms and two strains a few after 48 hours The latter, however, had disappeared after a further few days

TABLE 2

Antibacterial Effect of Phemerol and Thiomerosal Separately Against Pseudomonas Aeruginosa Strains

Strain no	Concentration				
	0	25 γ ml		100 γ ml	
		Phemerol	Thiomerosal	Phemerol	Thiomerosal
330	∞	∞	∞	0	0
660	∞	10^{2-3}	∞	0	0
661	∞	10^{2-3}	0	0	0
662	∞	10^{2-3}	0	0	0
663	∞	10^{3-4}	1*	3*	0
664	∞	10^{2-3}	0	0	0
665	∞	10^{2-3}	0	0	0
666	∞	10^{2-3}	2*	0	0
667	∞	10^{2-3}	3*	0	0
668	∞	10^{2-3}	0	1*	0
669	∞	10^{3-4}	10^{2-3}	0	0

The figures indicate the number of viable bacteria in 0 1 ml of test medium 48 hours after inoculation

* No viable organisms after 5 days contact

Thus, none of the Ps aeruginosa strains could be destroyed by phemerol but strain No 330 showed a rather pronounced resistance to phemerol (and also to thiomerosal) in a concentration of 25 γ per ml In the test medium at 4° C this concentration took ten days to destroy more than 98 per cent of the cells of No 330 However, if the concentration was doubled the pseudomonades were killed within one day

Thiomerosal As mentioned the plate counting method could be used to evaluate the antibacterial activity of thiomerosal for the Pseudomonas aeruginosa group The results of these experiments are shown in Table 2 together with those of the phemerol experiments In a concentration of 25 γ per ml thiomerosal killed completely all the organisms in eight out of eleven cultures within two to five days, one strain was partially inhibited and two strains not inhibited at all In this concentration thiomerosal had generally speaking a stronger antibacterial

25 γ per ml and at the appropriate growth temperatures, phemerol exerted a total bactericidal effect upon strains like E coli, B anitratum Ps diminuta, Klebsiella, Staph albus, B cereus and B subtilis Strains like Alcaligenes faecalis, Ps maltophilia, Ps fluorescens, Proteus vulgaris and Shigella sonnei were inhibited partially within the 48 hour observation period, while growth of Ps aeruginosa 330 and of Salmonella paratyphi B did not seem to be influenced at all

TABLE 1
Antibacterial Effect of Phemerol

Bacterial strain	Concentration							
	0		25 γ ml		50 γ ml		100 γ ml	
	M	C	M	C	M	C	M	C
E coli	+	∞		0			—	0
B anitratum	+	∞	—	0			—	0
Alc faecalis	+	∞	—	$10^{2\ 3}$	—	0	—	0
Proteus	-	$10^{4\ 5}$		$10^{3\ 4}$				0
Ps maltophilia	+	∞	-	$10^{2\ 3}$		0	—	0
Ps fluorescens	+	∞	-	$10^{2\ 3}$	—	0	—	0
Ps diminuta	—	$10^{3\ 4}$	—	0	—	0	—	0
Ps aeruginosa 330	+	∞	+	∞	—	0	—	0
Klebsiella	+	∞	—	0				0
Shig sonnei	+	∞		5			—	0
S paratyphi B	+	∞	+	∞			—	0
Staph albus	+	∞	—	0	—	0		0
B subtilis	+	∞	—	0				0
B cereus	+	∞	-	0				0

The figures indicate the number of viable bacteria in test medium 48 hours after inoculation

M = Macroscopic observation of growth in test medium
C = Plate counts from 0 1 ml of test medium

In a concentration of 50 γ per ml phemerol was tested against the following strains Alc faecalis, Ps maltophilia, Ps fluorescens, Ps diminuta and Ps aeruginosa 330, all of which were totally killed

In a concentration of 100 γ per ml, phemerol totally inhibited all the test organisms

In view of current problems regarding chemo resistant staphylococci it was considered to be of interest to investigate the sensitivity to phemerol of a group of Staphylococcus aureus strains Ten such cultures were inoculated into the test medium containing 25 and 100 γ of phemerol per ml and incubated at 37° C After three days none of the tubes showed any viable organisms when plated on broth agar

Also the Pseudomonas aeruginosa strains are known to represent a highly chemo-resistant group of organisms Since the culture of Ps aeruginosa (330) used in this series of experiments appeared to be the most resistant to the action of phemerol, it was decided to investigate

totally lost for several strains when the concentration was reduced to 25 γ per ml

This sharp decrease in the antibacterial activity of phemerol, which is much more pronounced than for the corresponding concentrations of thiomerosal, is quite characteristic. It is interesting to note that while 25 γ of phemerol per ml of test medium leaves the medium completely clear, 50 γ per ml gives some and 100 γ per ml very marked opalescence. Whether there is any connection between these observations has not been investigated.

TABLE 3

Antibacterial Effect of Phemerol and Thiomerosal Combined

Organism		D	C	M
B cereus	No preservative	10^{-6}	∞	++
	Thiomerosal 100 γ/ml	0		0
	Phemerol 25 γ/ml	0	0	0
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0		0
Staph aureus 12150	No preservative	10^{3-4}	10^{4-5}	++
	Thiomerosal 100 γ/ml	0		0
	Phemerol 25 γ/ml	0	0	0
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0		0
Ps aeruginosa 660	No preservative	10^{5-6}	10^{5-6}	++
	Thiomerosal 100 γ/ml	10^{1-2}	10^{2}	+
	Phemerol 25 γ/ml	10^{2-3}	3×10^{3}	+
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0	0	0

The figures indicate the number of viable bacteria in 1 ml of test medium 24 hours after inoculation estimated by (D) serial dilution in fluid thioglycolate medium and (C) plate counting

M = Macroscopic observation of growth in test medium

In a concentration of 100 γ per ml, phemerol seems to compete fairly well with thiomerosal, whereas in a concentration of 25 γ per ml its antibacterial activity is distinctly weaker than that of thiomerosal where comparison was made.

Considerable doubt has been raised concerning the value of mercurials as antiseptics. These are stated to act mainly bacteriostatically and to be inactive in the presence of —SH compounds and serum (*Fildes* 1940, *Morton et al* 1948, *Engley* 1950). However, as pointed out previously preservatives normally do not have to act upon actual bacterial cultures but under conditions more resembling the experimental conditions used in this study. It is therefore of great interest to note from these experiments that even in concentrations far below those normally used thiomerosal has a considerable bactericidal effect on the microorganisms selected for this study. This agrees well both with the rare

effect upon these organisms than phemerol However, the test strains differed more in their susceptibility to thiomerosal than to phemerol 100 γ of thiomerosal per ml of the test medium killed all the strains within 48 hours which was practically speaking also the case with phemerol

As mentioned previously the plate counting method could not be used for the other test strains For B cereus and Staphylococcus aureus in estimate of the antibacterial activity of thiomerosal was obtained by the technique described (see "Methods") These experiments revealed that growth of test strains of these bacteria in the test medium was inhibited by less than 1 γ of thiomerosal per ml The minimum bactericidal concentration as estimated from the thioglycolate tubes was more than 1 but less than 10 γ per ml for both strains after one day of incubation However, after three days it was the same as the minimum bacteriostatic concentration, *i e* less than 1 γ per ml

Thus, thiomerosal seems to have pronounced antibacterial properties under conditions similar to those under which the substance is used in practice In this connection, it must be taken into consideration that products preserved with thiomerosal are normally treated with heat (56° C for half an hour) which greatly enhances the antibacterial effect This has been verified under the experimental conditions used here

Action of Phemerol and Thiomerosal Combined

In view of the practical use of phemerol for our special purpose, it was felt to be of interest to investigate the antibacterial action of a combination of phemerol in a concentration of 25 γ per ml and thiomerosal in a concentration of 100 γ per ml

The experiments were carried out with the technique described Macroscopic observation of growth in tubes with test medium inoculated with the experimental strains, bacterial counting by the plate method, and serial dilution in fluid thioglycolate medium, gave the results summarized in Table 3 As previously established, B cereus and Staph aureus were totally killed by each of the two compounds in the concentrations mentioned, as was also the case with the combination *Ps aeruginosa 660 was only partially inhibited within the experimental* period by each of the substances but totally killed by the combination, thus indicating pronounced synergic action

DISCUSSION

The results of the present experiments indicate that in a concentration of 100 γ per ml the quaternary ammonium compound benze-
... m chloride (Phemerol®) has a bactericidal effect on the test orga-

totally lost for several strains when the concentration was reduced to 25 γ per ml

This sharp decrease in the antibacterial activity of phemerol, which is much more pronounced than for the corresponding concentrations of thiomerosal is quite characteristic It is interesting to note that while 25 γ of phemerol per ml of test medium leaves the medium completely clear, 50 γ per ml gives some and 100 γ per ml very marked opalescence Whether there is any connection between these observations has not been investigated

TABLE 3

Antibacterial Effect of Phemerol and Thiomerosal Combined

Organism		D	C	M
B cereus	No preservative	10^{5-6}	∞	++
	Thiomerosal 100 γ/ml	0		0
	Phemerol 25 γ/ml	0	0	0
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0		0
Staph aureus 12150	No preservative	10^{3-4}	10^{4-5}	++
	Thiomerosal 100 γ ml	0		0
	Phemerol 25 γ/ml	0	0	0
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0		0
Ps aeruginosa 660	No preservative	10^{5-6}	10^{4-5}	++
	Thiomerosal 100 γ/ml	10^{1-2}	10^2	+
	Phemerol 25 γ/ml	10^{2-3}	3×10^3	+
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0	0	0

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	Thiomerosal 100 γ/ml	0		0
B cereus	Phemerol 25 γ/ml	0	0	0
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0		0
	No preservative	10^{3-4}	10^{4-5}	++
	Thiomerosal 100 γ/ml	0		0
Staph aureus 12150	Phemerol 25 γ/ml	0	0	0
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0		0
	No preservative	10^{5-6}	10^{4-5}	++
	Thiomerosal 100 γ/ml	10^{1-2}	10^{7}	+
Ps aeruginosa 660	Phemerol 25 γ/ml	10^{2-3}	3×10^{3}	+
	Thiomerosal 100 γ/ml + Phemerol 25 γ/ml	0	0	0

The figures indicate the number of viable bacteria in 1 ml of test medium 24 hours after inoculation estimated by (D) serial dilution in fluid thioglycolate medium and (C) plate counting
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occurrence of contamination (presumably accidental) found in the routine sterility control of our preparations preserved with thiomerosal (*Scheibel & Bentzon* 1957) and with the fact that we have never recorded any clinical infection which could be ascribed to contamination of such products. *Brewer* (1950) also emphasizes the fact that the mercurials were first used as antiseptics because they prevented sepsis, and presents evidence to show that some of the mercurials (including thiomerosal) are just as capable of preventing infection as the common germicides.

It is also of interest that a synergic action between thiomerosal and phemerol could be demonstrated for a Pseudomonas aeruginosa culture which was partially resistant to the two compounds separately. Synergic action between mercurials and surface-active substances has also been reported by *Engley* (1950) and *Brewer* (1950). Thus, there does not seem to be any reason against using thiomerosal and phemerol combined, provided that they do not interfere with the action of the active substances in the solution to be preserved, an aspect which must be tested in each case.

SUMMARY

For evaluation of the antibacterial effect of a quaternary ammonium compound, Phemerol®, when used as preservative in toxoid vaccines, experimental conditions were adopted which equalled as much as possible the conditions of use.

In the concentrations 100 and 50 γ per ml phemerol showed a satisfactory bactericidal effect upon the test strains, whereas the concentration intended for use, 25 γ per ml, showed an insufficient antibacterial activity.

For comparison, thiomerosal was included in the experiments. In the concentration commonly used for preservation of biological products, 100 γ per ml, thiomerosal inhibited growth of all of the test cultures. the experiments indicated that the action was bactericidal under the conditions used, and not merely bacteriostatic, as is generally stated redarding the mercurials.

A synergic action between phemerol and thiomerosal was demonstrated.

REFERENCES

Brewer J H Mercurials as antiseptics Ann New York Acad Sci 53 211 1950

Engley F B Jr Evaluation of mercurial compounds as antiseptics Ann New York Acad Sci 53 197 1950

Fildes P The mechanism of the anti bacterial action of mercury Brit J Exper Path 21 67 1940

Morton, H F, North I L Jr & Engley F B The bacteriostatic and bactericidal ac[illegible] Med Ass.

[illegible] Fungicides 1957

Scheibel, I & Bentzon, M W Sterility control of sera and vaccines [illegible] Third Int Meet of Biol Stand, Opatija 2 6 Sept 1957

FROM RICKETTSIA & VIRUS DEPARTMENT STATENS SERUMINSTITUT, COPENHAGEN

STUDIES ON THE CHEMICAL NATURE OF ORNITHOSIS COMPLEMENT-FIXING ANTIGEN

By

PER MØLLER JØRGENSEN, E SØNDERGAARD and M VOLKERT

Received 14 viii 62

Previous reports (1, 2) from this department described a method for the production of an ornithosis complement-fixing antigen from infected yolk sacs Furthermore, chemical studies gave results which suggested that the antigen could be of a lipid nature and was connected to the phosphatides It is the purpose of this article to present continued studies on the physico-chemical characteristics of this ornithosis complement fixing antigen

MATERIAL AND METHODS

The source of antigen was yolk sacs infected with ornithosis virus The virus and the techniques used for antigen production and antigen titration were as described in the foregoing papers (1 2)

EXPERIMENTAL

Solubility in Organic Solvents

Effect of organic solvents on the ether acetone residue As the dry residue of acetone purified ether extracts from infected yolk sacs represented the purest and most potent antigen available, this product was the first choice for treatment with various organic solvents in order to investigate whether further improvements to the potency and purity of the antigen could be achieved Tests on saline solubility were included in order to have a basis for comparison

The results of the experiments are shown in Table I First it will be seen that it was possible to dissolve also this antigen in ether, even though the ether extraction was not complete and a considerable amount of the antigen remained in the residue Furthermore, a large number of solvents other than ether were able to extract the antigen but to varying degrees Benzene could extract all the ant[illegible] orm

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[illegible] the

same time caused a fall in titer This effect was even more pronounced with xylene Ethyl acetate could not extract anything and moreover had a deleterious effect on the antigen in the residue

TABLE 1
Effect of Organic Solvents on the Ether-acetone Residue

Solvent	Antigen in solvent	Antigen in residue
Saline	128	–
Ether	64	32
Benzene	256	2
Chloroform	128	1
Cyclohexane	128	2
Carbon tetrachloride	64–128	2
Xylene	32	0 1
Ethyl acetate	0	32 64
Ethanol, pure	0	16
Ethanol 75%	0	64
Ethanol 50%	128	32
Methanol pure	4	64 128

The effect of the alcohols ethanol and methanol depended on the concentration In pure alcohols the antigen was insoluble When the alcohols were diluted with water, some antigen went into alcohol-water phase The amount of antigen which was dissolved depended on the percentage of water in the alcohol-water mixtures, so that the most diluted alcohols extracted the most antigen

As was to be expected from the results previously described, the antigen was insoluble in acetone and furthermore acetone had no deleterious effect on the antigen in the residue

Effect of organic solvents on the ether extract residue from infected yolk sacs before acetone purification As it was found that organic solvents other than ether could dissolve the purified antigen, their effect on the antigen in an earlier phase of the production process was investigated First the solubility of the ether extract residue was examined The results are shown in Table 2 As was the case with the purified antigen, this product was completely dissolved in ether, benzene, cyclohexane, chloroform and saline, partly dissolved in xylene, carbon, tetrachloride and diluted alcohol but insoluble in acetone and in concentrated ethanol or methanol

Effect of organic solvents on infected boiled yolk sacs direct Using the antigen preparations employed in the foregoing experiments, several of the organic solvents could extract the antigen However, when extraction experiments were carried out direct on infected boiled yolk sacs exclusively, ether could dissolve as much antigen as was found in the control saline extract These results are shown in Table 3 It will also be seen from this table that the only other organic solvent which was able to extract some antigen was chloroform, but only small

amounts of antigen were found here That a weak antigenic titer could be demonstrated in the diluted ethanol extract could be expected if, as before, it is considered that the antigen is taken up by the water phase

Concerning the antigen titer in the residue after extraction, only acetone was found to leave the antigen undamaged

TABLE 2

Effect of Organic Solvents on the Ether treated Residue

	Antigen in solvent	Antigen in residue
Saline	32	
Ether	32	0
Benzene	64	0
Chloroform	32	0
Cyclohexane	64	0–1
Carbon tetrachloride	16	0
Xylene	16	0
Ethyl acetate	4	32
Ethanol pure		64 128
Ethanol 75%	0	128
Ethanol 50%	16	4
Methanol pure	1	64 128
Acetone	1	128

TABLE 3

Effect of Organic Solvents on Boiled Yolk Sac

	Antigen in solvent	Antigen in residue
Saline	128	
Ether	128	8 16
Benzene	8	16 32
Chloroform	32	4
Carbon tetrachloride	8	8 16
Ethyl acetate	0	16
Ethanol pure	0	16
Ethanol 75%	0	32
Ethanol 50%	4 8	32
Methanol pure	0	16
Acetone	0	64 128

Effect of organic solvents on the final ether and acetone purified antigen dissolved in saline The study shows that the solubility was completely different here, as will be seen from Table 4 When a saline antigen preparation was shaken with ether or secondary butyl alcohol, some antigen was taken up but most of it remained in the saline phase Moreover, when the saline antigen preparation was shaken with other organic solvents this procedure often resulted in formation of an intermediary gel phase which contained the antigen However by means of shaking

It w

antigen and solvent was carried out with great care in a separating funnel However, even with this method it was not possible to establish any definite relationship between the saline antigen preparation and the organic solvents

TABLE 4
Effect of Organic Solvents on Saline Suspended Antigen

	Antigen in solvent	Antigen in residue
Ether	128	512
Secondary buthyl alcohol	64	256
Benzene	0 1	256–512
Chloroform	1	512
Cyclohexane	0	512
Carbon tetrachloride	1 2	256
Xylene	4 8	512
Ethyl acetate	8	64
Petroleum ether	0	256

Stabilizers

Since the transfer of antigen from saline to ether was rather poor and also inconsistent, attempts were made to increase the ether solubility by means of the addition of lecithine and normal yolk to the saline preparations These attempts were unsuccessful

Purification Results

The main aim of the solubility experiment was to obtain a further purification of the final antigen As all the saline suspended antigen had a milky, cloudy or opalescent appearance, an idea of the purity of the preparations could be obtained by photometric measurements In our experiments we used a Hilger photometer with filter no 49

The most striking result was achieved when ether extract residue from boiled yolk sacs was treated with acetone and the clearest preparations were obtained if the extraction was carried out at 56° C The preparations thus obtained were only slightly opalescent in saline Methanol and ethanol extraction of the ether residue also caused some purification but not nearly as well as acetone Methanol and ethanol extraction of the ether-acetone residue gave a clearer antigen but also caused a reduction in antigenic potency

Other organic solvents which were able to extract the antigen from the different sources were never found to give as good results as ether This was so even when the residue from these extracts was treated with acetone or alcohols

Repeated extractions with any organic solvent including ether and acetone, always caused a reduction in antigenic titer The deleterious effect on the antigen of such repeated treatments was greater than any purification achieved

As mentioned above, acetone extraction of the ether extracted residue from boiled yolk sacs gave the purest antigen However, acetone does more than purify, one extraction also enhances the antigenic potency It was found repeatedly that the antigenic titer after the acetone treatment had risen to at least twice the titer level Moreover, our experiments also showed repeatedly that acetone was able to remove anti complementary characteristics which might sometimes be in the preparations

Solubility of the Antigen in Saline

The extent of the solubility of the antigen in saline was determined by means of high speed centrifugation in a Spinco centrifuge

It was found that the more rapid the centrifugation, the better the antigen precipitated, and that the antigen could be precipitated completely from water phase, though this necessitated very vigorous treatment, *viz* centrifugation at 40,000 r p m for one hour

Some antigenic effect may be left in the supernatant despite this treatment but generally it can be stated that the antigen can be precipitated

Filtration of the Antigen Solution

Filtration of the saline suspension of the antigen through Seitz filter No 3 showed that filtration was easy and the filtrate clear The filter disc was comminuted after the experiment and eluated with saline Neither the filtrate nor the eluate from the filter disc contained antigen This can only be interpreted to the effect that the antigen is adsorbed to the filter fibers and that this adsorption is so strong that it is not possible to liberate the antigen from it again with saline

Effect of Acid and Bases on the Antigen

Antigen suspensions were treated with acids and bases on waterbath at 100° C for 30 minutes, after which the antigen was brought back to neutral point before titration Hydrochloride acid in concentrations from 0 1 n to 4 n was used It was found that the antigen was acid labile since it was destroyed by the weakest concentration of hydrochloride acid used (0 1 n)

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with 4 n NaOH ... effect was seen

Enzyme Experiments

A *Experiments with trypsin* These showed that treatment with 0 05 per cent trypsin at 37° C for two days and with 0 5 per cent trypsin at

37° C for 30 minutes had no effect on the antigenic activity This result would suggest that the active component is not a protein substance

B *Experiments with lipases* The first experiment was performed with pancreatin solubile Novo, which is a fairly impure lipase product containing amylase The concentrations were 0 5, 5, and 10 per cent The titers became lower the higher the enzyme concentration

Since the changes observed in the antigen might have been due either to the lipase or to the impurities in the product, the experiment was repeated with a lipase produced at the Department of Biophysics, Statens Seruminstitut This product was purer than the commercial one The experiment was carried out at room temperature

The same effect was observed again, *viz* a fall in the antigen titer but never total destruction of the antigenic activity, even when high enzyme concentrations were used (see Table 3)

Desalination

It was found that the antigen could be precipitated by ammonium sulphate Precipitation commenced at half saturation and was complete at full saturation

Potassium Periodate

An experiment by which to determine the effect of KJO_4 on the antigen resulted in the disappearance of the antigenic activity The effect was very strong and was evident down to a total KJO_4 concentration of 1/32 satured solution with exposure for 30 minutes at 100° and for 24 hours at 37°

Chromatography

A number of experiments were performed using both column and paper chromatography, in the hope that it would be possible to achieve a separation of the antigen components and thus approach elucidation of the active principle However, these attempts met with no success As will be seen from Table 4, it was not possible to find any organic solvent which could give even a fairly constant relationship between solvent and antigen suspension

Electrophoresis

Electrophoretic studies were carried out on both the usual routine antigen and on antigen purified with methanol The result of these experiments was that all the particles in the suspension (solution) migrated *i e* that they were electrically charged In addition, the antigenic activity was uniformly distributed over all the components Thus it was not possible by this method either to achieve separation of the active antigenic factor from possible inactive subsidiary substances

TABLE 5

Effect of Pancreas Lipase on Ornithosis Antigen Produced by Ether-acetone Method

Control	0.1% lipase				1% lipase				10% lipase			
Unboiled 512	Boiled			Unboiled	Boiled			Unboiled	Boiled			Unboiled
Boiled 256	0 min	60 mins	24 hrs	24 hrs	0 min	60 mins	24 hrs	24 hrs	0 min	60 mins	24 hrs	24 hrs
Water phase	512	256	256	512	256	256	128	512	64	64	64	128
Supernatant	512	256	256	512	256	256	128	512	0	0	0	64
Sediment	16	8	8	32	8	8	64	16	64	64	128	64

Dry Matter Determination

This was performed on the purest and strongest antigen preparation available The ether extracted, acetone purified antigen was suspended in distilled water and then freeze-dried The dry matter content was then 2 3 mg per ml in a preparation with an antigen titer of 1024

DISCUSSION AND SUMMARY

The data presented here have shown that it is possible to dissolve the complement-fixing ornithosis antigen from infected yolk sacs in a number of lipid solvents However, the extracting effect of these solvents is highly dependent on the physical state of the antigen and on the presence of lipoid impurities The antigen was insoluble in acetone, methanol, and ethanol and, when treated with these, some purification was achieved The best results were obtained with acetone which in addition to purifying was also found to cause an enhancement of the antigenic potency, and to be able to remove anti-complementary substances Methanol and ethanol had no such advantage, on the contrary, treatment with these alcohols caused a decrease in the antigenic titer Of the lipoid solvents, none had a stronger extracting effect than ether, but on certain antigen preparations other solvents were just as good The conclusion to be drawn from all experiments must be that ether is the best choice for extracting purposes, and that acetone treatment of the ether residue gives the purest and most potent antigen Therefore, for practical purposes the method described by *Volkert & Møller Christensen* (1) must still be considered the simplest and most effective for antigen preparation

The pronounced affinity of the antigen for many lipoid solvents supports the supposition of *Volkert & Møller Christensen* that the active antigenic principle is of phosphatide nature or at least strongly connected to lipids in the phosphatide group

The fact that the antigen is insoluble in methanol shows that it is different in character from cardiolipin

By means of centrifugation of boiled yolk sacs in a Spinco centrifuge *Møller Christensen* (3) found that the antigen is located just below the fat which forms as a layer at the top after centrifugation This would suggest that the antigen becomes attached to the fat drops with which it rises The similar experiments carried out with antigen suspension washed in ether and acetone showed that the antigen forms sediment though only after strong treatment The reason for this could be that the ether-acetone treatment liberates the antigen from the lipid particles so that it is able to form sediment

The resistance of the antigen to trypsin indicates that the active component is not a protein

The decrease in titer which occurred in the lipase experiments supports the supposition that the antigen contains a lipoid component

The effect of periodate on the antigen was very great but periodate is so strong an oxidizing agent that that result cannot be taken as an indication that carbohydrate is the active complement fixing antigen component

However, there is good reason for assuming that the antigen is a lipo polysaccharide

Unfortunately, chromatography and electrophoresis did not provide information regarding the part played by the individual components in the antigenic activity

Dry matter analysis showed that the content of dry matter was very small, *viz* 0 1 mg ml, *i e* 0 2 mg dry matter per yolk sac

REFERENCES

1 *Møller Christensen P & M Volkert* The Phosphatide Antigen The Virus Antigen and Methods for their Preparation Acta path et microbiol scandinav 37 211 218 1955

2 *Møller Christensen P & M Volkert* The Nature of the Virus Antigen and its Relation to the Phosphatide Antigen Acta path et microb ol scandinav 37 219 224 1955

3 *Møller Christensen P* Ornithosis A Study of Virus and Antigen Copenhagen 1957 68

THE IN VITRO ACTIVITY OF STAPHYLOMYCIN

Spectrum, Routine Sensitivity Tests, and Cross-Resistance

By

V FROLUND THOMSEN

Received 3 ix 62

The new antibiotic staphylomycin[1], now available in Denmark, is produced by a species of streptomyces closely related to streptomyces virginiae The fungus was first isolated in 1954 from a sample of Belgian soil by *de Somer & van Dijck* who in 1955 described the compound under the name of Antibiotic no 899 It is closely related to the generally known streptogramin and oleandomycin (*Vanderhaeghe et al* 1957, *Charney et al* 1953)

Staphylomycin is composed of three components, viz M_I, M_{II}, and S, of which the M-components have been found the most active against Staphylococci, whereas the C-component is the most effective against Bacillus subtilis M_I is found to represent 75 per cent, S about 5 per cent, and M_{II} no more than 5 per cent of the compound (*Vanderhaeghe et al* 1957) Staphylomycin is a yellowish powder, only slightly soluble in water (0 6 per mille at pH = 6), but readily soluble in organic solvents as *e g* methanol and ethanol

Usually staphylomycin is used for local application as a powder, an ointment, or a solution, but the antibiotic is available also in capsules for peroral administration The antibiotic is found to possess a low toxicity and to be well tolerated by mice in single doses of 1000 mg per kg (*de Somer & van Dijck* 1955)

Actually, the antibiotic is effective if administered by mouth, but investigations by *de Somer & van Dijck* (1955) revealed a marked discrepancy between the demonstrability of staphylomycin in serum and its protecting effect, *i e* experimental animals were protected against experimental infections, although no staphylomycin was demonstrable in their sera

Dons & Foged (1961) have treated 37 patients suffering from infections caused by penicillin-resistant staphylococci by oral or local administration or a combination of the two and found satisfactory response to treatment in 33 of these cases Similar results were obtained by *de Somer & van de Voorde* (1957) who treated 50 patients with

1 Staphylomycin was placed at my disposal by courtesy of Lindenburg & Riemer Ltd Copenhagen Denmark

staphylococcus infections Minor secondary effects were seen only in 4 of these 87 cases and in no case were side reactions of a serious nature

Staphylomycin is most effective against the gram positive bacteria The growth of staphylococci is inhibited by the antibiotic in concentrations of about 0 2 μg per ml growth of pneumococci in concentrations of about 0 07 μg per ml and of streptococci in similar concentrations It is active also against the gram positive rods though not to the same extent With one exception viz the Haemophilus pertussis staphylomycin seems to be ineffective against the gram negative rods (*de Somer & van Dijck* 1955)

Hitherto no cross resistance has been demonstrable with one or more of the known antibiotics Staphylococci known to be resistant to most of the generally used antibiotics were found to be highly sensitive to staphylomycin (*de Somer & van Dijck* 1955 *Dons & Foged* 1961)

In the present investigation the main object has been to determine the activity of staphylomycin against various bacterial groups The purpose has been (1) to specify the spectrum of the compound (2) to establish a method by which to determine the bacterial sensitivity to staphylomycin and (3) to evaluate any cross resistance between staphylomycin and other antibiotics

MATERIAL AND METHOD

Using the plate-dilution method the 50 per cent inhibitory staphylomycin concentration has been tested against 175 strains belonging to 19 genera In addition the inhibition zones of some of the same strains have been measured by the agar diffusion method on the basis of the findings a converting scheme has been prepared to be used in routine sensitivity tests with he agar diffusion method

The Plate Dilution Method

The examinations were carried out on 5 per cent blood agar poured into 9 cm Petri dishes Immediately before the agar solidifies an adequate quantity of staphylomycin is added to the substrate and thoroughly mixed The quantity to be added is calculated to make the final concentration in blood agar of staphylomycin form two fold dilution steps from 50 to 0 2 μg per ml and an antibiotic free control plate

Gram negative rods	1 1000
Staphylococci gram positive rods and streptococcus faecalis	1 100
Other streptococci and pneumococci	1 10

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cause the former is less dependent on the density ... concentration (MIC) is determined

The Agar Prediffusion Method

The pre diffusion technique used at Statens Seruminstitut (Frølund Thomsen (to [illegible]

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cent blood agar without peptone, but with the addition of 1 per cent of glucose poured into large Petri dishes (14 cm) with a thickness of layer of 10 mm (*Lund* 1955) The discs are placed on the substrate and the plate is kept for 20 hours at *room temperature (20° C) with a view to establishing a stable concentrationgradient* Subsequently the discs are removed and the plate inoculated by 3 drops of the above culture dilution using a bent glass rod for the spreading The inhibition zones are determined simultaneously on the same plate against benzylpenicillin dimethoxy phenylpenicillin, tetracyclin chloramphenicol and erythromycin

Experiments with the Plate Dilution Method

Using the plate dilution method the activity of staphylomycin is determined on gram-positive cocci and rods as well as on gram negative rods (Tables 1–3)

Table 1 gives data of the gram-positive cocci among which Staphylococcus aureus has been the object of the greatest interest, a total of 63 strains are examined 44 of these (70 per cent) are inhibited by ≤ 0 2 µg per ml, 17 strains (27 per cent) are inhibited by 0 2–0 4 µg per ml, and 2 strains only (3 per cent) require higher inhibitory concentrations *viz* 0 8 and 1 6 µg per ml 7 of 8 examined Staphylococcus albus strains are inhibited by ≤ 0 2 µg per ml, whereas one strain is found to be inhibited by concentrations within 0 2–0 4 µg per ml

TABLE 1

The Activity of Staphylomycin against 103 Strains of Gram Positive Cocci The Table Shows the Number of Strains Inhibited by the Various Concentrations Strains on Border Values are Recorded in the Sensitive Groups

Species	50% inhibitory concentration in µg per ml				
	3 2 1 6	1 6 0 8	0 8 0 4	0 4 0 2	0 2
Staph aureus		1	1	17	44
Staph albus				1	7
Pneumococcus					8
Strep haem Gr A				1	7
Strep viridans				4	4
Strep faecalis	1	7			

All of the 8 Pneumococcus strains are inhibited by 0 2 µg per ml which also applies to 7 of the 8 haemolytic streptococcus strains group A, whereas one strain is *inhibited by 0 4 µg per ml 4 of 8 Streptococcus* viridans are inhibited by 0 02 µg per ml the remaining 4 by 0 2 0 4 µg per ml Streptococcus faecalis is found to be more resistant 7 strains are found to be inhibited by concentrations from 0 8 1 6 µg per ml, 1 from 1 6–3 2 µg per ml

TABLE 2

The Activity of Staphylomycin against 16 Strains of Gram Positive Rods (Further Explanation cf. Table 1)

	50% inhibitory concentration in µg per ml		
	0.8 0.4	0.4 0.2	< 0.2
Listeria monocyt.		8	
Corynebact. dipht.			6
Bact. cereus	1		
Bact. subtilis		1	

Table 2 records the activity of staphylomycin on a group of 16 gram positive rods all of which are sensitive. 8 Listeria monocytogenes strains are inhibited by 0.2–0.4 µg per ml and 6 Corynebacterium diphteriae strains are inhibited by ≤ 0.2 µg per ml. One Bacillus cereus strain is inhibited by 0.8 µg per ml and one Bacillus subtilis by 0.4 µg per ml.

The very weak inhibitory effect on gram negative rods of staphylomycin is apparent from Table 3. A total of 72 strains are examined. Effects are demonstrable only in one E. coli, one Salmonella and one Shigella strain but the effects are of no practical interest.

TABLE 3

The Activity of Staphylomycin against 72 Strains of Gram Negative Rods (For Further Explanation cf. Table 1)

	50% inhibitory concentration in µg per ml			
	> 25	25 12.5	12.5 6.2	6.2 3.1
Pseudom. aeruginosa	8			
Esch. coli	7			1
Klebsiella	8			
Salmonella	7	1		
Shigella	7		1	
Prot. mirabilis	4			
Prot. vulgaris	4			
Prot. morganii	8			
Prot. rettgeri	8			
Providencia	8			

Routine Determination of Bacterial Sensitivity to Staphylomycin Using the Pre Diffusion Method

The [illegible]
on the [illegible]
which [illegible] concentrations below one third to one fifth of the serum concentration after normal doses are considered sensitive, whereas strains which are not inhibited by concentrations ob

The Agar Prediffusion Method

The pre diffusion technique used at Statens Seruminstitut (Frølund Thomsen (to be publ.)) has been applied for determinations with the agar-diffusion method. Filter paper discs with diameters of 6 mm are dipped into a 1.9 per mille alcoholic staphylomycin solution thus *holding 50 μg in each disc. The substrate is a 10 per* cent blood agar without peptone, but with the addition of 1 per cent of glucose poured into large Petri dishes (14 cm) with a thickness of layer of 10 mm (*Lund* 1955). The discs are placed on the substrate and the plate is kept for 20 hours at room temperature (20° C) with a view to establishing a stable concentrationgradient. Subsequently the discs are removed and the plate inoculated by 3 drops of the above culture dilution using a bent glass rod for the spreading. The inhibition zones are determined simultaneously on the same plate against benzylpenicillin, dimethoxy *phenylpenicillin, tetracyclin, chloramphenicol and erythromycin.*

Experiments with the Plate Dilution Method

Using the plate dilution method the activity of staphylomycin is determined on gram-positive cocci and rods as well as on gram-negative *rods (Tables 1–3).*

Table 1 gives data of the gram-positive cocci among which Staphylococcus aureus has been the object of the greatest interest, a total of 63 strains are examined. 44 of these (70 per cent) are inhibited by ≤ 0.2 μg per ml, 17 strains (27 per cent) are inhibited by 0.2–0.4 μg per ml, and 2 strains only (3 per cent) require higher inhibitory concentrations *viz.* 0.8 and 1.6 μg per ml. 7 of 8 examined Staphylococcus albus strains are inhibited by ≤ 0.2 μg per ml, whereas one strain is found to be inhibited by concentrations within 0.2–0.4 μg per ml.

TABLE 1

The Activity of Staphylomycin against 103 Strains of Gram Positive Cocci. The Table Shows the Number of Strains Inhibited by the Various Concentrations. Strains on Border Values are Recorded in the Sensitive Groups

Species	0% inhibitory concentration in μg per ml				
	3.2–1.6	1.6–0.8	0.8–0.4	0.4–0.2	0.2
Staph. aureus		1	1	17	44
Staph. albus				1	7
Pneumococcus					8
Strep. haem. Gr. A				1	7
Strep. viridans				4	4
Strep. faecalis	1	7			

All of the 8 Pneumococcus strains are inhibited by 0.2 μg per ml, which also applies to 7 of the 8 haemolytic streptococcus strains group A, whereas one strain is inhibited by 0.4 μg per ml. 4 of 8 Streptococcus viridans are inhibited by 0.02 μg per ml, the remaining 4 by 0.2–0.4 μg per ml. Streptococcus faecalis is found to be more resistant, 7 strains are found to be inhibited by *concentrations from 0.8–1.6 μg per ml,* 1 from 1.6–3.2 μg per ml.

TABLE 2

The Activity of Staphylomycin against 16 Strains of Gram Positive Rods (Further Explanation cf. Table 1)

	50% inhibitory concentration in µg per ml		
	0.8–0.4	0.4–0.2	≤0.2
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Corynebact. dipht.			6
Bact. cereus	1		
Bact. subtilis		1	

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Pseud. aeruginosa	8			
Esch. coli	7			1
Klebsiella	8			
Salmonella	7	1		
Shigella	7		1	
Prot. mirabilis	4			
Prot. vulgaris	4			
Prot. morganii	8			
Prot. rettgeri	8			
Pr. vulgaris	8			

Routine Determination of Bacterial Sensitivity to Staphylomycin Using the Pre Diffusion Method

The classification of bacteria into different sensitivity groups is based on the concentrations of antibiotics in serum and body fluids. Strains which in vitro are inhibited by concentrations below *one third to one fifth* of the serum concentration after normal doses are considered sensitive, whereas strains which are *not* inhibited by concentrations ob-

tainable in clinical routine are considered resistant In this study serum- and tissue concentrations are not known and hence the classification can be preliminary only and rather arbitrary In Statens Serum institut the groups recorded in Table 4 are adopted preliminary

It is seen from the table that strains inhibited by ≤ 1 µg per ml are considered sensitive to staphylomycin because satisfactory clinical effects have been obtained in infections caused by staphylococci known to be inhibited by concentrations of about 0 2 µg per ml

TABLE 4

Classification of Bacteria into Different Sensitivity Groups According to Sensitivity to Staphylomycin and the Relationship between 50 per Cent Inhibitory Concentration and the Zone Size in the Pre Diffusion Method

	50% inhibitory concentration with plate-dilution method	Sensitivity group	Diameter of inhibition zone with pre-diffusion method In mm*)
Sensitive	< 1 µg per ml	+ + +	≧ 30 mm
Moderately sensitive	1– 5 µg per ml	+ +	29 17 mm
Relatively resistant	5–20 µg per ml	+	16– 7 mm
Resistant	>20 µg per ml	0	≦ 6 mm

* The diameter is determined in discs (6 mm) holding 50 µg of staphylomycin on 10 per cent blood agar without peptone after pre diffusion for 20 hours at 20° C

TABLE 5

The Sensitivity of 8 Staphylococcus aureus as Determined by the Pre Diffusion Method Recording Inhibition Zones in mm

Strain	Staphylomycin 20 mcg*)	Benzyl penicillin 1 U	Dimethoxyphenylpenicillin 20 mcg	Tetracyclin 100 mcg	Chloramphenicol 30 mcg	Erythromycin 10 mcg
Staph aureus 124	33	0	38	0	0	0
226	26	35	34	0	0	0
227	37	0	43	0	34	0
228	32	0	34	49	30	0
229	33	0	30	0	0	0
230	34	0	40	39	0	0
231	34	0	40	0	0	0
232	30	0	43	0	34	0

* Quantity of antibiotic in disc

The pre diffusion method is calibrated as follows a number of strains are selected in which the 50 per cent inhibitory concentration has been determined by the plate dilution method, the sensitivity of the same strains is determined simultaneously using discs containing 100, 50, and 25 µg after a period of pre-diffusion of 20 hours The corresponding values are plotted in a diagram for each disc content and the optimal

disc content is chosen by comparison of the diagrams 50 μg per disc is found to give small inhibition zones with relatively resistant strains which are inhibited by 10–20 μg per ml, and zones of ≥ 30 mm with strains which are inhibited by ≤ 1 μg per ml Hence, in the prevailing circumstances this content must be considered most adequate (Fig 1)

By means of the stipled horizontal lines which indicate the limits between the sensitivity groups the corresponding limits in inhibition zones are determined (cf Table 4) Although the points are distributed along a slightly curved line the fixed straight line will give a sufficiently reliable correspondance between the inhibitory concentration and the diameter of the inhibition zone

Correlation of Sensitivity to Staphylomycin and to five other Antibiotics as Determined by the Pre-Diffusion Method

A correlation between the sensitivity of staphylococci to benzylpenicillin and staphylomycin is of great practical interest A total of 63

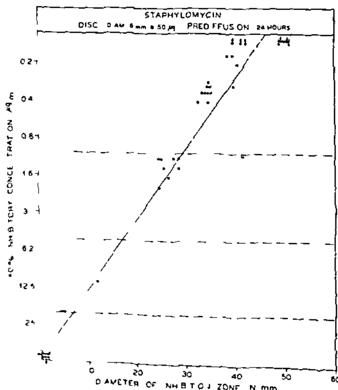


Fig 1

The correlation between values obtained by the plate dilution method and the prediffusion method Each point indicates one strain The broken lines show limits between sensitivity groups

tainable in clinical routine are considered resistant. In this study serum- and tissue concentrations are not known and hence the classification can be preliminary only and rather arbitrary. In Statens Serum institut the groups recorded in Table 4 are adopted preliminary.

It is seen from the table that strains inhibited by ≤ 1 μg per ml are considered sensitive to staphylomycin because satisfactory clinical effects have been obtained in infections caused by staphylococci known to be inhibited by concentrations of about 0.2 μg per ml.

TABLE 4

Classification of Bacteria into Different Sensitivity Groups According to Sensitivity to Staphylomycin and the Relationship between 50 per Cent Inhibitory Concentration and the Zone Size in the Pre-Diffusion Method

	50% inhibitory concentration with plate dilution method	Sensitivity group	Diameter of inhibition zone with pre-diffusion method (in mm*)
Sensitive	< 1 μg per ml	+ + +	$\geqq$ 30 mm
Moderately sensitive	1–5 μg per ml	+ +	29–17 mm
Relatively resistant	5–20 μg per ml	+	16–7 mm
Resistant	>20 μg per ml	0	$\leqq$ 6 mm

* The diameter is determined in discs (6 mm) holding 50 μg of staphylomycin on 10 per cent blood agar without peptone after pre-diffusion for 20 hours at 20° C.

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230	34	0	40	39	0	0
231	34	0	40	0	0	0
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* Quantity of antibiotic in disc.

The pre-diffusion method is calibrated as follows: a number of strains are selected in which the 50 per cent inhibitory concentration has been determined by the plate dilution method; the sensitivity of the same strains is determined simultaneously using discs containing 100, 50 and 25 μg after a period of pre-diffusion of 20 hours. The corresponding values are plotted in a diagram for each disc content, and the optimal

Considering the pros et cons with a view to the mainly topical use of the antibiotic it must be admitted that it hardly ever will be used in universal treatments of severe, general infections and, moreover that no cross resistance with the known antibiotics seems to be involved Whether a development of resistance to other antibiotics accompanies the development of resistance to staphylomycin cannot be deducted from this, but such development may be anticipated On the assumption that resistance to staphylomycin need not involve simultaneous resistance to other antibiotics, it must be considered adequate for local use Within these limits of indication staphylomycin must be received as a valuable supplement to the number of antibiotics available for the topical treatment of local infections

The routine determination of bacterial sensitivity may be carried out by a diffusion as well as a dilution method The velocity of diffusion of the compound being rather low, inhibition zones in tests using sensitive strains will be relatively small Parallel with features known from other compounds the correlation between the dilution and diffusion methods may be improved here also by pre diffusion of plates (*Frolund Thomsen* 1962) Parallel with our increasing knowledge of serum and tissue concentrations, the arbitrary classification into groups of sensitivity has to be changed

SUMMARY

The in vitro activity of staphylomycin is determined by the plate dilution assay against 175 strains belonging to 19 different genera

The inhibitory concentration of staphylomycin against Staphylococcus aureus is found to be about 0 2 µg per ml, the variation of the inhibitory concentration in the different strains is found to be negligible

Staphylomycin affects almost exclusively the gram positive bacteria and is particularly active against the gram positive cocci

No cross resistance of staphylomycin has been demonstrable with penicillin G tetracyclin, chloramphenicol and erythromycin

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REFERENCES

Charney J Fisher W P Curran C Machlowitz R A & Tytell A A Streptogramin a new antibi tic Antibi tics & Chemotherapy ...
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Staphylococcus aureus strains was tested for sensitivity against these two antibiotics, 36 of these strains were found to be resistant to penicillin, 27 where sensitive to penicillin. The sensitivity to staphylomycin in these two groups was of the same order.

Table 5 shows the data of sensitivity in 8 Staphylococcus aureus strains which are resistant to erythromycin. It will be noted that sensitivity to staphylomycin is independent of the sensitivity to erythromycin as well as to penicillin, tetracyclin, and chloramphenicol. Nothing can be said about dimethoxyphenylpenicillin, as all of the strains were sensitive.

DISCUSSION

In the treatment of bacterial infections, in particular infections caused by the multiresistant bacteria, the introduction of any new and active antibiotic is of great value. Staphylomycin represents a drug against infections caused by Staphylococcus aureus, and it has been effective even against such staphylococci as are found resistant to other antibiotics. So far no exhaustive reports have been published on the spectrum of staphylomycin.

The results obtained in this study show inhibitory concentrations of staphylomycin against staphylococci of ≤ 0.4 μg per ml (70 per cent of the strains are inhibited by ≤ 0.2 μg per ml). This is in accord with the few results previously reported (*Dons & Foged* 1961) and besides it has been shown that the sensitivity of staphylococci to staphylomycin is independent of sensitivity to other antibiotics as *e.g.* penicillin G, *tetracyclin, chloramphenicol, and erythromycin*.

Staphylomycin is active almost exclusively against the gram positive bacteria, in which regard it is comparable with the newer antibiotics as *e.g.* vancomycin, ristocetin, and oleandomycin.

The sensitivity to staphylomycin of the 63 Staphylococcus aureus strains is rather uniform. With the dilution method two of the 63 strains were found to be more resistant than the remaining 61. When the diffusion method was used one of these presented a sensitivity which equalled sensitivities of the others and the results obtained with the dilution method may thus be due to experimental errors, the other strain was found to have a higher resistance when assayed with the diffusion method and should be considered more resistant. Otherwise the sensitivity in the various genera is found only to differ slightly.

The question is now whether the application of the antibiotic is *justified* or whether side reactions may be anticipated which contraindicate such application. The main hazard, however, involved in the introduction of a new antibiotic is that it may produce resistant strains either directly during treatment or as a result of a selection on a long view whereby it, sooner or later, may loose its effectivity and contribute to an unfortunate *alteration of the bacterial flora*.

How seriously these hazards are to be taken is a matter of opinion

FROM THE DEPARTMENT OF PATHOLOGY (HEAD J CLEMMESEN M.D.)
THE FINSEN INSTITUTE AND RADIUM CENTER, COPENHAGEN

METABOLISM OF H.A.1 TUMOUR CELLS

Glycolysis and Crabtree Effect
Sensitivity of Respiration to Oxygen Tension

By

BENT L. SØRENSEN

Received 21 viii 62

The demonstration of differences in the metabolism of malignant and normal cells may afford guidance for chemotherapeutic experiments Conversely, the induction of metabolic tumour-cell characteristics in normal cells may form the basis of a study of the mechanism of carcinogenesis, which is the object of the present study

An investigation of this nature however, is only possible by means of an experimental method that can reproduce with sufficient certainty the metabolic differences between malignant and benign tissues Therefore, the study of these differences must be the primary object

In the present study of the metabolic characteristics of malignant cells the author used H A 1 tumour cells In a subsequent paper the results will be applied in experiments on carcinogenesis

The H A 1 ascites tumour was found in 1956 by *Iversen,* who described its chromosomal characteristics in 1958a Heterologous transplantation from mice to rats was attempted by *Iversen* in 1958b and by *Iversen & Sørensen* in 1960, and the growth data of the tumour were analysed by *Sørensen* in 1962

The ability of malignant tissues as distinct from benign tissues to form lactic acid under aerobic and anaerobic conditions was demonstrated by *Warburg* in 1923, and it was shown by *Crabtree* in 1929 that addition of glucose inhibits the respiration (Crabtree effect) of malignant cells while only a few benign tissues show such effect The reaction of H A 1 tumour cells to addition of glucose is studied in the following, and the explanation of the Crabtree effect will be discussed in relation to the Pasteur effect

Osgood & Krippaehne demonstrated in 1955 that the growth of leukaemic cells *in vitro* had an optimal oxygen tension *Kieler* (1957) found the maximum respiration of leukaemic cells at a gas phase of 10 per cent O_2, whereas the respiration of normal leukocytes does not depend upon the oxygen tension These investigations were carried out

de Somer P & Dijck P van A preliminary report on antibiotic no 899 a streptogramin like substance Antibiotics & Chemotherapy 5 632 1955

de Somer P & van de Voorde H First clinical investigations with staphylomycin (antibiotic number 899) in fifty cases Antibiot Med & Clin Ther 4 786 1957

Thomsen V Frolund Correlation of the plate dilution method to the agar diffusion method (disc and tablet method) with a special view to the importance of pre diffusion Acta path et microbiol scandinav 54 107 1962

Thomsen V Frolund Resistensbestemmelse med pre diffusionsmetoden to be publ

Vanderhaeghe H van Dijck P Parmentier G & de Somer P Isolation and properties of the components of staphylomycin Antibiotics & Chemotherapy 7 606 1957

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with the "Cartesian diver respirometer" (*Linderstrøm Lang* 1943), and the present author has attempted to reproduce Kieler's findings in leukaemic cells and later in Yoshida ascites cells, (*Kieler* 1960a) with H A 1 tumour cells in an ordinary Warburg respirometer

METHOD AND MATERIAL

The H A 1 tumour cells were aspirated from 3 month old Bagg mice 8 to 11 days after transplantation Haemorrhagic ascites was discarded Differential counts of the ascites fluid showed that 85 per cent of the cells were tumour cells 12 per cent polymorphonuclear granulocytes and 3 per cent histiocyte like cells No mast cell were observed The average concentration of cells was 125 000 per cubic mm of ascites with a coefficient of variation of 20 per cent Counting was carried out in a Burker Turk haemocytometer *after dilution with the medium used* The ascites containing the cells was diluted at least 50 times so that the ascitic fluid made up a maximum of 2 per cent of the cellular suspension used in the experiments Warburg's indirect method (*Warburg* 1924) was used for determining glycolysis Crabtree effect and Kieler effect Moreover the Crabtree effect was investigated using Warburg's direct method (*Warburg* 1923) The cell counts per Warburg flask ranged from 3×10^6 to 13×10 The average of 5 investigations showed that 1 mg H A 1 tumour cells as dry substance is made up of $3\ 19 \times 10^6$ cells

The medium used for the indirect Warburg experiments was a modified Ringer Locke solution (*Parker* 1950) which contains per litre KCl 5 C338 mMol CaCl 2 162 mMol $NaHCO_3$ 14 5 mMol NaCl 129 C357 mMol Tris (hydroxymethyl) amino *methane (Tris) 30 0 mMol Moreover penicillin 0 121 g and streptomycin 0 050 g* were added per litre This solution contains per litre 330 osmotic milliequivalents By adding other compounds or removing compounds already present the amount of NaCl may be regulated to keep the osmotic concentration constant The same solution without $NaHCO_3$ was used for the direct Warburg experiments

Tris was described by *Gomori* in 1946 The pH of the buffer is adjusted to 7 4 The capacity of 3 ml of buffer with HCO_3 and 5 per cent CO in the gas phase was calculated in the way that approx 12×10^{-6} equivalents acid = approx 300 mm^3 CO corresponding to the maximum capacity of the manometers alters pH 0 1 into the acid direction Without bicarbonate and CO the corresponding capacity is only about 3×10^{-6} equivalents = approx 70 mm^3 CO

Owing to the ability of the Tris buffer for *acid and CO_2 retention* the manometric lactic acid determination was corrected as advocated by *Umbreit Burris & Stauffer* 1957 Experiments showed that the Tris buffer retained 54 per cent of the produced lactic acid and 10 per cent of the respiratory CO

With a view to measuring *respiratory activity at various oxygen tensions* the author assessed the uncertainty in single experiments by means of parallel experiments with different oxygen tension in the gas phase thus ascertaining the significance limit on differences determined by the present method The variance on two single determinations was found to be 0 1380 At the 5 per cent significance limit and with 7 degrees of freedom two single determinations therefore differ by a maximum of $2\ 4 \times 0\ 37 = 0\ 89$

The gas phase containing 5 per cent CO was bubbled through the bicarbonate containing buffers for 5 hours at 37° by means of a fine glass filter with a view to physical equilibrium

RESULTS

Glycolysis

On the assumption that the RQ value of the H A 1 tumour cells is 0 86 the glycolytic CO production was calculated on the basis of the total computed CO production at a glucose concentration of 50 mMol and gas phases of 95 per cent O + 5 per cent CO (designated as aerobic) and 95 per cent N + 5 per cent CO (+ 0 15 per cent O_2) (designated as anaerobic) respectively The values found are listed in Table 1

TABLE 1

Respiration (Q_{O_2}) and Glycolysis of the H A 1 Tumour Cells under Aerobic Conditions ($Q_I^{O_2}$) and under Anaerobic Conditions ($Q_I^{N_2}$), Stated per Cell per Hour in mm³ × 10⁻⁶

	Q_{O_2}	$Q_I^{O_2}$	$Q_I^{N_2}$	P F	M O Q
	5 9	8 8	16 8	8 0	4 1
	4 0	4 4	16 2	11 8	8 9
	3 1	4 2	8 6	4 4	4 3
	7 6	9 9	18 7	8 8	3 5
	5 3	8 2	17 2	9 0	5 1
	2 1	2 7	4 4	1 7	2 4
Mean	4 7	6 4	13 7	7.3	4 7

Absolute Pasteur Effect (P F) $= Q_I^{N_2} - Q_I^{O_2}$ Meyerhof's Oxidation Coefficient (M O Q) $= 3 \times \frac{PF}{Q_{O_2}}$ Tris bicarbonate buffer 50 mMol glucose Warburg's indirect method

Conversion of the mean values in Table 1 to mg dry weight gives the values shown in Table 2

In other words, each time the tumour cells take up 1 mol O_2 under aerobic conditions 1 36 mol lactic acid is formed

Under anaerobic conditions 43 7 mm³ CO_2 is liberated by the produced lactic acid per mg dry weight per hour This corresponds to 0 001951 mMol of lactic acid, and to this end 0 0009755 mMol of glucose = 0 17559 mg of glucose is consumed Thus, under anaerobic conditions 1 mg of glucose will be consumed in about 6 hours

As a control on the manometric method the lactic acid formation was [illegible] formation [illegible] nge of the [illegible] manometrically On the average, the anaerobic glycolysis amounts to 118 per cent of the aerobic glycolysis

TABLE 2

Mean Values from Table 1 Converted to per mg Dry Weight Values Stated in mm³

Q_{O_2}	$Q_I^{O_2}$	$Q_I^{N_2}$	P F	M O Q
15 0	20 4	43 7	23.3	4 7

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The medium used for the indirect Warburg experiments was a modified Ringer Locke solution (*Parker* 1950) which contains per litre KCl 5 6338 mMol CaCl 2 1625 mMol $NaHCO_3$ 14 5 mMol NaCl 129 6357 mMol Tris (hydroxymethyl) amino methane ("Tris") 30 0 mMol Moreover penicillin 0 121 g and streptomycin 0 050 g were added per litre This solution contains per litre 336 osmotic milliequivalents By adding other compounds or removing compounds already present the amount of NaCl may be regulated to keep the osmotic concentration constant The same solution without $NaHCO_3$ was used for the direct Warburg experiments

"Tris" was described by *Gomori* in 1946 The pH of the buffer is adjusted to 7 4 The capacity of 3 ml of buffer with HCO_3 and 5 per cent CO_2 in the gas phase was calculated in the way that approx 12×10^{-6} equivalents acid = approx 300 mm^3 CO_2 corresponding to the maximum capacity of the manometers alters pH 0 1 into the acid direction Without bicarbonate and CO_2 the corresponding capacity is only about 3×10^{-6} equivalents = approx 70 mm^3 CO_2

Owing to the ability of the Tris buffer for acid and CO_2 retention the manometric lactic acid determination was corrected as advocated by *Umbreit Burris & Stauffer* 1957 Experiments showed that the Tris buffer retained 54 per cent of the produced lactic acid and 10 per cent of the respiratory CO_2

With a view to measuring respiratory activity at various oxygen tensions the author assessed the uncertainty in single experiments by means of parallel experiments with different oxygen tension in the gas phase thus ascertaining the significance limit on differences determined by the present method The variance on two single determinations was found to be 0 1380 At the 5 per cent significance limit and with 7 degrees of freedom two single determinations therefore differ by a maximum of $2\ 4 \times 0\ 37 = 0\ 89$

The gas phase containing 5 per cent CO_2 was bubbled through the bicarbonate containing buffers for 5 hours at 37° by means of a fine glass filter with a view to physical equilibrium

RESULTS

Glycolysis

On the assumption that the RQ value of the H A 1 tumour cells is 0 86, the glycolytic CO_2 production was calculated on the basis of the total computed CO_2 production at a glucose concentration of 50 mMol and gas phases of 95 per cent O_2 + 5 per cent CO_2 (designated as aerobic) and 95 per cent N_2 + 5 per cent CO_2 (+ 0 15 per cent O_2) (designated as anaerobic) respectively The values found are listed in Table 1

is the same irrespective of the presence of glucose Taken from Fig 1 the oxygen uptake with 50 mMol of glucose, hour by hour in relation to the oxygen uptake without glucose shows the following inhibition or stimulation caused by 50 mMol of glucose (Table 4)

TABLE 4

Oxygen Uptake of the H A 1 Tumour Cells (mm³ × 10⁻⁶) without Glucose and with 50 mMol of Glucose

	Q_O		B in per cent of A
	A	B	
	0 mM glucose	50 mM glucose	
1st hour	6 6	2 9	44
2nd hour	4 1	3 3	80
3rd hour	3 3	4 0	121
4th hour	2 1	3 9	186

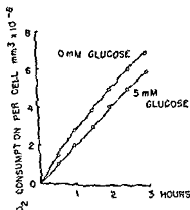


Fig 2

Effect of 5 mMol of glucose on respiration of H A 1 tumour cells
Transbüffer Direct Warburg method Atmospheric air

When the glucose concentration is 5 mMol the respiration curves show the course illustrated in Fig 2

Comparison of the magnitude of the O_2 uptake with 5 mMol of glucose with the O_2 uptake without the presence of glucose hour by hour gives the values shown in Table 5 representing 4 experiments

It appears that using glucose in a concentration of 5 mMol the oxygen uptake will not have been stimulated at the end of a few hours as was seen at a glucose concentration of 50 mMol In the first 3 hours inhibition amounted to about 20 per cent whereupon the O_2 uptake increased to the endogenous level but not above This indicates that the

TABLE 3

Lactic Acid Formation by H A 1 Tumour Cells Determined Chemically 50 mMol of Glucose Values Given per Hour in mm³ CO_2

	Gas phase	Aerobic	Anaerobic	PF
Q_l	per cell (× 10^{-6})	4 6	10 9	6 3
	per mg dry weight	14 7	34 8	20 1

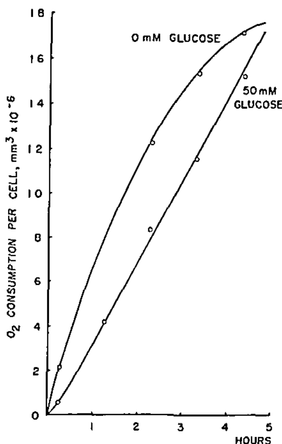


Fig 1

Effect of 50 mMol of glucose on respiration of H A 1 tumour cells Tris bicarbonate buffer Indirect Warburg method 95 per cent O_2 + 5 per cent CO_2

Crabtree Effect

The O_2 uptake of H A 1 tumour cells at a glucose concentration of 50 mMol, was compared with the O_2 uptake under endogenous conditions The respiration curve is presented in Fig 1 which shows that during most of the first hour the presence of glucose will inhibit the oxygen uptake which subsequently continues following a straight line Without the presence of glucose the oxygen uptake starts at a high rate, but shows an increasing fall At the end of about two hours the Qo_2

TABLE 6

Endogenous Respiration of H A 1 Tumour Cells in the First Experimental Hour Stated per Cell in mm³ × 10⁻⁶ at Varying O_2 Tensions

Experiment No	Q_{O_2}				
	Per cent O_2 in the gas phase				
	5	10	20	50	95
1	2.4 (77)		3.1 (100)		2.3 (74)
2		2.7 (159)	1.7 (100)		
3			4.3 (100)	2.5 (58)	
4	2.2 (183)		1.2 (100)		
5		1.4 (100)	1.4 (100)		
6		2.8 (140)	2.0 (100)		
7			1.4 (100)		1.1 (79)
8	0.7 (54)	1.5 (115)	1.3 (100)		
9			1.3 (100)	1.3 (100)	
10	1.0 (38)		2.6 (100)	1.3 (50)	
11			1.5 (100)	1.9 (127)	
12	2.1 (140)		1.5 (100)	1.2 (80)	
13	1.8 (62)		2.9 (100)	3.4 (117)	
14	2.4 (50)		4.8 (100)	3.5 (73)	
Mean of percentages	85	129	100	86	77

The figures in brackets indicate the Q_{O_2} in per cent of Q_{O_2} at 20 per cent O_2. *In addition to the O_2 percentage stated all of the air mixtures contain 5 per cent CO_2.* The remainder was N_2. Tris bicarbonate *buffer*. Warburg's indirect method.

The bottom line in Table 6 shows that with 10 per cent O_2 in the gas phase the endogenous *respiration* of the H A 1 tumour cells is greatest, inhibition *occurring at a* higher as well as lower oxygen content. However, this tendency could not be *demonstrated* in every experiment. Assessment of the individual *experiments* when the oxygen percentage is lowered from 10 (or from 20) *to* 5 per cent (a difference in Q_{O_2} of 0.9 being considered significant) shows,

glucose has been consumed and that the function now measured is the endogenous respiration The immediate inhibition by 5 mMol of glucose is below that of 50 mMol of glucose

According to *Quastel & Bickis* (1959) the Crabtree effect × 2 P O = aerobic glycolysis $(Q_{O_2} — Q_{O_2}\ (\text{gluc}))\times 2\ P/O = Q_I^O$

As P/O was found to be 3,

$$\frac{Q_I^O}{Q_{O_2} — Q_{O_2}\ (\text{gluc})} = 6 \qquad (I)$$

TABLE 5

Oxygen Uptake of the H A 1 Tumour Cells (mm³ × 10 6) without Glucose and with 5 mMol of Glucose Direct Warburg Method Atmospheric Air

	Q_O								B in per cent of A				
	A				B								
	0 mMol glucose				5 mMol glucose								
Exper No	1	2	3	4	1	2	3	4	1	2	3	4	Mean
1st hour	1 7	2 2	2 6	1 7	1 4	1 8	1 9	1 7	82	82	73	100	84
2nd hour	1 6	2 2	2 4	1 9	1 4	1 8	1 5	1 4	88	82	63	74	78
3rd hour		2 2	2 2	1 7		1 8	1 7	1 5		82	77	88	82
4th hour				1 7				1 5				88	88
5th hour				1 5				1 5				100	100
6th hour				1 6				1 5				94	94

This ratio may be expected if the ATP production in the tumours is constant, whether glucose is present or not, the ATP deficit caused by the Crabtree effect being replaced by the aerobic glycolysis

The glycolysis values in Table 1 are from the 2nd hour of the experiments in which the respiration has again become rectilinear The mean value for glycolysis is 6 4 × 10 ⁶ Table 4 shows that in the second hour a Crabtree effect of *4 9 × 10 ⁶ — 3 9 × 10 ⁶ = 1 0 × 10 ⁶* These values inserted into (I) make

$$\frac{6\ 4}{1\ 0} = 6\ 4$$

The Effect of Oxygen Tension on the Respiration of H A 1 Tumour Cells

Experimental procedure The respiration of H A 1 tumour cells with 20 per cent O_2 in the gas phase in each experiment was compared with the respiration in one or two other gas phases 5 per cent, 10 per cent, 50 per cent or 95 per cent O_2

The results obtained in the first experimental hour may be seen from Table 6

DISCUSSION

Glycolysis

The pronounced ability of tumour cells to form lactic acid was demonstrated by *Warburg* in 1923 In 1924 *Warburg, Posener & Negelein* found that under anaerobic conditions the Flexner-Jobling rat carcinoma consumed 12 4 per cent per minute of its own dry weight in added glucose and 7 per cent under aerobic conditions, correspondingly, Jensen's rat sarcoma showed a consumption of glucose of 16 per cent and 10 per cent under anaerobic and aerobic conditions respectively The findings for H A 1 tumour cells in the present study correspond exactly to these early results

Studying the Ehrlich ascites carcinoma, *Warburg* in 1956, found an anaerobic glycolysis of 70 mm³ CO_2 and aerobic of 30 per mg dry weight With the Krebs-2 mouse ascites tumour *Yushok* (1959) found an aerobic glycolysis of 22 and anaerobic glycolysis of 44 53 The absolute glycolysis values for the H A 1 tumour cells also correspond to the results of previous investigations into ascites tumours

Normal tissues generally show a low aerobic and anaerobic glycolysis An exception is formed by embryonic tissue which has a high anaerobic glycolysis but no notable aerobic glycolysis (*Warburg, Posener & Negelein* 1924 *Burk* 1939, *Fujita* 1928, *Warburg & Kubowitz* 1927) However *Villee* (1954) found aerobic glycolysis with human foetal tissue which showed no signs of decreasing from the 10th to the 40th week of gestation

A number of non embryonic tissues have shown a marked anaerobic glycolysis This applies to the grey matter of the brain in rats (*Warburg, Posener & Negelein* 1924) and to bone marrow as well as to leukocytes The medulla of the guinea-pig kidney also has a high anaerobic glycolysis (20 30) combined with an aerobic glycolysis of 10 15 (*Dickens & Weil Malherbe* 1936) In the case of myeloid bone marrow *Warren* (1943) found aerobic glycolysis at 9 and anaerobic glycolysis at 22 Re[illegible]

[illegible] *Posener & Negelein* 1924) In general, it may be said that the normal non embryonic tissues show anaerobic glycolysis, sometimes appreciable, but aerobic glycolysis is rarely demonstrated except in very low values

Crabtree Effect

In 1929 Crabtree demonstrated that a 10 mMol glucose inhibits the respiration of Jensen's rat sarcoma by about 16 per cent In ascites tumours this Crabtree effect was demonstrated by *Kun, Talalay & Williams Ashman* (1951) who found a 50 per cent inhibition of the respiration on addition of glucose to Ehrlich ascites carcinoma In normal tissues this effect of glucose has been found only exceptionally, *e g* in

—a significant inhibition in 3 out of 7 experiments (Experiments 10, 13 and 14)

—a non-significant inhibition in 2 out of 7 experiments (Experiments 1 and 8)

—a non-significant stimulation in one out of 7 experiments (Experiment 12)

—a significant stimulation in one out of 7 experiments (Experiment 4)

At an oxygen content increasing beyond 10 per cent and 20 per cent O_2 the respiration showed:

—a significant inhibition in 4 out 13 experiments (Experiments 2, 3, 10, and 14)

—a non-significant inhibition in 5 out of 13 experiments (Experiments 1, 6, 7, 8, and 12)

—no difference in 2 out of 13 experiments (Experiments 11 and 13)

—a non-significant stimulation in 2 out of 13 experiments (Experiments 11 and 13)

—a significant stimulation in 0 out of 13 experiments

Only the two experiments showing stimulation of the respiration at 5 per cent O_2 militate against maximum respiration at 10 per cent O. In these two experiments the maximum respiration occurs at an oxygen content below 10 per cent.

Only 2 experiments militate against an inhibition of the respiration at an oxygen content of more than 20 per cent in the gas phase.

Thus, during the first experimental hour only 4 out of 14 experiments militated against the respiration being maximal at 10 per cent O_2 in the gas phase and inhibited at lower as well as higher oxygen levels.

Examples of typical respiration curves at various oxygen tensions are given in Fig. 3.

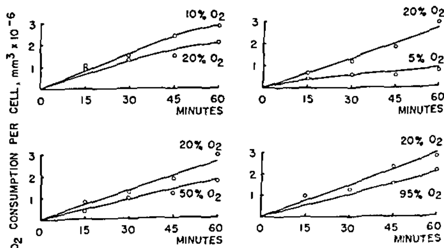


Fig. 3

Effect of various oxygen concentrations on the respiration of H A 1 tumour cells. Indirect Warburg method.

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renal tissue from rats and in bovine articular cartilage by *Rosenthal, Bowie & Wagoner* (1940), and in retinal tissue from newborn rabbits by *Cohen* (1957)

The Pasteur effect and the Crabtree effect are demonstrable in H A 1 tumour cells In contradistinction to the Pasteur effect the Crabtree effect seemingly has a closer coupling between the oxidative and glycolytic phosphorylation, the energy—in the form of ATP—lost through the respiratory inhibition being replaced by the ATP gained by the synthesis through glycolysis This has been demonstrated by *Quastel & Bickis* (1959) and confirmed, in the case of H A 1 tumour cells, in the present study On the other hand, a calculation as above shows for the Pasteur reaction a gain of energy in the form of ATP by the respiration which is 3 to 6 times greater than that lost through glycolysis When the Pasteur effect is 7 3 and the oxygen uptake 4 7 per cell per hour, *and when 1 mol of ATP is formed per mol of lactic acid and 6 mol of* ATP per mol of oxygen taken up, the gain of energy in the H A 1 tumour cells upon transition from anaerobic to aerobic conditions will be $6 \times 4\ 7 = 28\ 2$ ATP as compared with the glycolytic loss of 7 3 ATP, *i e* a 3 9 times substitution of the lost glycolytic energy The same calculation shows that under aerobic conditions, with glucose as substrate, the ratio oxidative/glycolytic energy is $6 \times Qo_2/Q_l^{O_2} = 28\ 4/6\ 4 = 4\ 4$ In other words Under aerobic conditions the glycolysis contributes by 18 5 per cent to the total energy production, calculated as ATP synthesis Similarly, the total anaerobic energy production in the H A 1 tumour cells makes up only about 40 per cent of the total aerobic energy However, *Quastel & Bickis* (1959) found that the anaerobic ability of tumours to incorporate amino acids in protein was equal to or greater than under aerobic conditions, the energy released through glycolysis under anaerobic conditions being just as active as the energy developed in the presence of oxygen In contradistinction, normal tissues and embryonic tissue could utilize the aerobic energy better than the anaerobic energy for the incorporation of amino acids in proteins

The Pasteur effect and the Crabtree effect indicate the existence of a regulation of the metabolism in normal cells and tumour cells The Crabtree effect—the inhibition of the respiration due to glycolysis—has been called the reverse Pasteur reaction, the Pasteur reaction being an inhibition of glycolysis due to respiration

The present position as regards an explanation of these effects is apparent from reviews by *Racker et al* and *Chance et al* who have recently advanced the views to be reported below

Studying ascites tumour cells *Racker, Wu & Alpers* (1960) found that hexokinase and phosphofructokinase, which produce ADP from ATP in the glycolytic system, had a low activity as compared with phosphoglycerokinase and pyruvatekinase which synthesize ATP from ADP in the glycolytic system In other words, they claimed that the ADP phos-

phorylating enzymes of the glycolysis represent a real, competitive factor against the oxidative phosphorylation of the mitochondria. This is perhaps the explanation why addition of glucose inhibits the respiration and why tumour cells have such a marked production of lactic acid.

Alterations in the inorganic phosphate of the ascites cells occur parallel with the intensity of glycolysis. The uptake of extracellular phosphate was found to be too slow to supplement the phosphorylation of glycolysis. Furthermore, respiring mitochondria compete with glyceraldehyde 3-phosphate dehydrogenase for inorganic phosphate when glucose is present. The explanation of how sufficient inorganic phosphate is procured for the glycolysis might then be that various syntheses requiring ATP liberate phosphorus for the glycolysis.

Thus glycolysis and respiration compete for ADP as well as inorganic phosphate, for which reason glycolysis can inhibit the respiration (the Crabtree effect), and the oxidative phosphorylation in the tumour cells is not sufficient to prevent aerobic synthesis of lactic acid. Instead of Warburg's assumption that the tumour formation is due to damage to the respiratory apparatus, it might be imagined that the primary change will be an increased synthesis of glyceraldehyde 3-phosphate dehydrogenase, since a high activity *of these enzymes increases the* aerobic glycolysis.

Immediately after addition of glucose to Ehrlich ascites tumour cells, *Chance* (1960) found increased respiration, explained by the ADP formed by glucose phosphorylation stimulating the phosphorylation of the mitochondria. Thus, contrary to the view of Racker et al. the respiration *is then able* to compete with glycolysis for ADP. The increased respiration is associated with a large consumption of glucose, thus the hexokinase activity must be high— contrary to Racker's statement. This 1st *period during* which respiration and glucose phosphorylation is increased *lasts for about* one minute *and is followed by* a second period of almost totally inhibited respiration *and glycolysis*. The explanation is that the ATP synthesized by oxidative phosphorylation is kept spatially separated from and *inaccessible to the* hexokinase reaction. Furthermore the investigations showed *that no* pyruvate is formed during the first period, and hence no ATP, synthesized by glycolysis, is available for the hexokinase reaction. *At the end of* a few minutes the inhibition of the glycolysis and the *respiration subsides, and the third period* follows with low respiration and high glycolysis corresponding to the classical Crabtree effect. *Thus, in the* second period we have a new phenomenon representing a metabolic regulation of the tumour cells.

Lastly it may be *mentioned* that *Kieler* (1960b) has shown that 1 per cent CO_2 in the gas phase *may inhibit* or abolish the Crabtree effect in Yoshida ascites tumour cells. *The effect* has been explained by a CO_2 fixation to phosphoenolpyruvate (*Wood & Werkman* 1938), forming oxaloacetate which is a *metabolite* in Krebs' cycle. This results *in an*

introduction into Krebs' cycle of glycolytic products, by-passing pyruvate and acetyl-coenzyme A, which stimulates the respiration of the cells. When phosphoenolpyruvate is converted into oxaloacetate instead of into pyruvate, the glycolysis saves ADP for which the glycolytic and respiratory systems must be assumed to compete as alredy mentioned. The ADP thus saved is then available for the oxidative phosphorylation, and the respiration increases. Instead of ADP, inosine diphosphate is found to act as a phosphate acceptor by synthesizing oxaloacetate as described above (*Utter & Kurahashi* 1954).

Effect of Oxygen Tension

Osgood & Krippaehne (1955) demonstrated that the growth of leukaemic cells *in vitro* was dependent upon the oxygen tension, and *Kieler* (1957) showed that the respiration of leukaemic cells was at a maximum when the gas phase contained 10 per cent O_2, while the respiration of normal leukocytes did not depend upon the oxygen concentration in the gas phase. *Kempner* (1939) found that in many normal tissues low oxygen tensions caused low RQ values, which might indicate that chiefly fats are burned. Accordingly, *Kieler* (1960a), studying Yoshida ascites tumour cells which show maximum respiration when the gas phase contains 20 per cent O_2, found that addition of butyrate at this oxygen concentration stimulated the respiration more than at 10 per cent O_2, while at 30 per cent O_2 there was an inhibitory effect upon the oxygen uptake of the tumour cells. When *Ciaranfi* (1938) failed to find a respiratory stimulatory effect of fatty acids in tumour tissue the reason is that his experiments were carried out in pure oxygen. The use of non-optimal oxygen tensions for the consumption of fat may also explain the findings by *Weinhouse, Allen & Millington* (1953) who in experiments in pure oxygen demonstrated that a neoplastic liver burns fatty acids at a slower rate than a normal liver, and stated (*Weinhouse, Millington & Wenner* 1951) that fatty acids in tumour tissue are broken down quantitatively and qualitatively just as in a representative group of normal tissues. *Medes & Weinhouse* (1958), furthermore, found that glucose in 100 per cent O_2 may inhibit the consumption of fat, glucose being preferred by Ehrlich ascites carcinoma cells, while glucose in a normal liver, according to *Weinhouse, Millington & Friedman* (1949) gives rise to an increased fat oxidation.

In the light of the most recent experiments performed at a physiological oxygen tension, it may therefore be presumed that fats are not only normal nutrients, but the main endogenous substrate for the energy of tumour cells.

Because of technical difficulties in calibrating apparatuses for the measurement of oxygen tension in tissues, the literature gives only little information regarding the oxygen tension at which the ascites tumour cells in tumour-bearing mice exist. On the basis of investiga-

tions on Ehrlich ascites tumour, *Deschner & Gray* (1959) found the oxygen tension on the peritoneal surface to be about 15 mm Hg in mice breathing atmospheric air and 40 mm Hg when they were in pure oxygen In all cases the oxygen tension falls with the distance from the peritoneal surface, tumour cells 0 15 mm away from it existing under anaerobic conditions This means at all times 80 per cent of the tumour cells

The essential problem in connection with the oxygen tension optimal for the tumour cells is probably not the rôle of oxygen tension in the tumour genesis (cf *Warburg* 1956a, *Weinhouse, Warburg, Burk & Shade* 1956) *Kieler* (1960a) assumes that if the conversion of pyruvate to active acetate were inhibited in tumour cells, Krebs' cycle can utilize, instead, metabolites from the breakdown of fatty acid whose conversion to active acetate is not inhibited at a physiological oxygen tension The finding that C^{14}-labelled glucose in tumour cells is recovered in respiratory CO_2 need not militate against the presence of a "bottleneck" between the glycolytic system and Krebs' cycle, as the glycolytic products may be transferred to Krebs' cycle via the Wood & Werkman reaction (*Wood & Werkman* 1938)

According to the results stated, the optimal oxygen tension for the endogenous respiration of the H A 1 tumour cells must be assumed to range from 10 to 20 per cent O_2 in the gas phase This corresponds to Kieler's finding in the case of human leukaemic cells in the Cartesian diver and in the case of Yoshida ascites tumour cells under the same conditions It should be mentioned, however, that a few of the experiments failed to confirm this predominant tendency Under the assumption that the high respiration at the oxygen tension concerned is due to fatty acids being burnt unhindered, it is incalculable whether, in cases where the respiration is not maximal at 10–20 per cent O_2, the tumour cells have contained a carbohydrate depot in the form of glycogen which is preferred to fat as an endogenous substrate for the respiration of tumour cells This was found by *Medes, Thomas & Weinhouse* (1960) in the case of Ehrlich ascites tumour cells To a great extent these cells will consume palmitate for the respiration, but if glucose were offered in small quantities palmitate would be spared and glucose burned instead However a poss[illegible]
hydrate in the H A 1 tumour [illegible]
ed as the tumour cells have [illegible]
the reading of the results has started Nevertheless, variations in these cells content of nutrients must be expected to influence the experimental results Furthermore, the factors which influence the share of the amino acids in the measured respiration have not been elucidated Only it is known that ammonia may be formed In the case of Jensen's rat sarcoma this corresponds to the fact that half the respiration was due to breakdown of amino acids (*Dickens & Greville* 1933) Finally, it must be mentioned that the present method fails to demonstrate

definitely differences in Qo_2 under 0 9, while the technique used by Kieler is considerably more sensitive

SUMMARY

The glycolytic factors relating to H A1 tumour cells were investigated Under aerobic as well as under anaerobic conditions, the values representing the glycolysis of the tumour cells were found to be characteristic of ascites tumours when compared with the findings of previous authors Under anaerobic conditions the cells were found to have, in 6 hours, a consumption of glucose for lactic acid production which corresponded to their own dry weight Inhibition of respiration due to glycolysis (Crabtree effect) was demonstrated in experiments on H A 1 tumour cells The energy synthesis lost through the respiratory inhibition appears to have been replaced by increased glycolysis

The rôle of oxygen tension in the respiration of tumour cells was demonstrated by Kieler in 1957 The investigations into H A 1 tumour cells show that under endogenous conditions the respiration is at a maximum when the gas phase contains 10 20 per cent O_2 which, according to Kieler's studies, appears to be characteristic of tumour cells At increasing oxygen tensions the respiration of the tumour cells is inhibited, presumably because of an inhibition of fat breakdown Despite a predominant tendency, the results of the H A 1 tumour cell experiments were rather varied This may be due to a deficient knowledge as regards the nature of the endogenous nutritional depot of the tumour cells Owing to its greater sensitivity, 'the Cartesian diver respirometer' must be considered more adequate for the demonstration of slight differences in the respiration of cells than the ordinary Warburg apparatus used in the present experiments

REFERENCES

Burk D A colloquial consideration of the Pasteur and neo Pasteur effects Cold Spring Harbor Sympos Quant Biol *7* 420 459 1939

Chance B A digital computer representation of chemical and spectroscopic studies on chemical control of ascites tumour cell metabolism Amino acid protein and cancer biochemistry p 191–212 1960 Ac Press

Chiaranfi E On the ability of tumor tissue to oxidize the fatty acids *in vitro* Am J Cancer *32* 561 564 1938

Cohen I H Glucose inhibition of respiration in the developing retina Fed Proc *16* 165 166 1957

Crabtree H G Observations on the carbohydrate metabolism of tumours Bioch J *23* 536 545 *1929*

Deschner E E & Gray L H Influence of oxygen tension on X ray induced chromo ... tumor cells irradiated *in vitro* Radiation Res

... sm of normal and tumour tissue IX Ammonia ... 7 1123 1133 1933

Dickens F & ... ... abolism of normal and tumour tissue XIV A note on the metabolism *of medulla of kidney Bioch J 30 659 660 1936*

Fujita A Über den Stoffwechsel der Korperzellen Biochem Ztschr *197* 175 188 1928

Gomori G Buffers in the range of pH 6 5 to 9 6 Proc Soc Exper Biol & Med 62 33 34 1946
Iversen H G Human tumours in cortisone treated mice Brit J Cancer *10* 472–480 1956
Iversen H G Transplantation from man to mouse of exudates containing tumour cells Brit J Cancer *12* 210–221, 1958a
Iversen H G The maintenance and adaptability of a mouse ascites tumour (H A 1) in roentgen irradiated rats Brit J Cancer *12* 370 377, 1958b
Iversen H G & Sorensen B L Immunological tolerance in heterologous tumour

Etiology Pathophysiology and Treatment p 215 226 1957 Ac Press
Kieler J Studies on oxidative metabolism of the Yoshida ascites tumour cells Acta Unio internat contra cancrum *16* 1079–1085 1960a
Kieler J Influence of CO_2 tension on the respiration of Yoshida ascites tumor cells J Nat Cancer Inst *25* 161–176 1960b
Kun E Talalay P & Williams Ashman H G Studies on the Ehrlich ascites tumor I The enzymic and metabolic activities of the ascitic cells and the ascitic plasma Cancer Res *11* 855–863 1951
Linderstrom Lang K On the theory of the cartesian diver micro respirometer Compt Rend Lab Carlsberg Sér chim 24 333 398 1943
Medes G Thomas A J & Weinhouse S Metabolism of neoplastic tissue XV Oxidation of exogenous fatty acids in Lettré Ehrlich ascites tumor cells J Nat Cancer Inst 24 1 12 1960
Medes G & Weinhouse S Metabolism of neoplastic tissue XIII Substrate competition in fatty acid oxidation in ascites tumor cells Cancer Res *18* 352 359 1958
Osgood E E & Krippaehne M L The gradient tissue culture method Exper Cell Res 9 116–127 1955
Parker R C Methods of tissue culture p 77 1950 Hoeber
Quastel J H & Bickis I J Metabolism of normal tissues and neoplasms *in vitro* Nature 183 281 286 1959
Racker E Wu R & Alpers J B Carbohydrate metabolism in ascites tumor and HeLa cells Amino acids proteins and cancer biochemistry p 175–189 1960 Ac Press
Rosenthal O Bowie M A & Wagoner G On the interdependence of respiration and glycolysis Science 92 382 383 1940
Sorensen B L Biological data of the H A 1 tumour Acta path et microbiol scandinav 56 13 28 1962
Umbreit W W Burris R H & Stauffer J F Manometric techniques p 97 101 1957 Burgess
Utter M F & Kurahashi K Mechanism of action of oxalacetic carboxylase J Biol Chem 207 821 841 1954
Villee C A The intermediary metabolism of human fetal tissues Cold Spring Harb Sympos Quant Biol *19* 186–193 1954
Warburg O Versuch an Überlebendem Carcinomgewebe (Methoden) Biochem Ztschr *142* 317 333 1923
Warburg O Verbesserte Methode zur Messung der Atmung und Glykolyse *Biochem Ztschr* *152* 51–63 1924
Warburg O
Warburg O
Warburg O
Chem
Warburg O Posener K & Negelein E Über den Stoffwechsel der Carcinomzelle Biochem Ztschr *152* 309 344 1924
Warren C O Tissue metabolism studies on bone marrow Consideration in relation to tumor metabolism Cancer Res 3 621 625 1943
Weinhouse S Allen A & Millington R H Metabolism of neoplastic tissue V Fatty acid oxidation in slices of transplanted tumors Cancer Res 13 367–371 1953
Weinhouse S Millington R H & Friedman B The effect of carbohydrate on the oxidation of fatty acids by liver slices J Biol Chem *181* 489 497 1949

definitely differences in Q_{O_2} under 0.9, while the technique used by Kieler is considerably more sensitive.

SUMMARY

The glycolytic factors relating to H A1 tumour cells were investigated. Under aerobic as well as under anaerobic conditions, the values representing the glycolysis of the tumour cells were found to be characteristic of ascites tumours when compared with the findings of previous authors. Under anaerobic conditions the cells were found to have, in 6 hours, a consumption of glucose for lactic acid production which corresponded to their own dry weight. Inhibition of respiration due to glycolysis (Crabtree effect) was demonstrated in experiments on H A 1 tumour cells. The energy synthesis lost through the respiratory inhibition appears to have been replaced by increased glycolysis.

The rôle of oxygen tension in the respiration of tumour cells was demonstrated by *Kieler* in 1957. The investigations into H A 1 tumour cells show that under endogenous conditions the respiration is at a maximum when the gas phase contains 10–20 per cent O_2 which, according to Kieler's studies, appears to be characteristic of tumour cells. At increasing oxygen tensions the respiration of the tumour cells is inhibited, presumably because of an inhibition of fat breakdown. Despite a predominant tendency, the results of the H A 1 tumour cell experiments were rather varied. This may be due to a deficient knowledge as regards the nature of the endogenous nutritional depot of the tumour cells. Owing to its greater sensitivity, "the Cartesian diver respirometer" must be considered more adequate for the demonstration of slight differences in the respiration of cells than the ordinary Warburg apparatus used in the present experiments.

REFERENCES

Burk D A colloquial consideration of the Pasteur and neo Pasteur effects Cold Spring Harbor Sympos Quant Biol 7 420 459 1939

Chance B A digital computer representation of chemical and spectroscopic studies on chemical control of ascites tumour cell metabolism Amino acid protein and cancer biochemistry p 191 212 1960 Ac Press

Chiaranfi F On the ability of tumor tissue to oxidize the fatty acids *in vitro* Am J Cancer 32 561 564 1938

Cohen I H Glucose inhibition of respiration in the developing retina Fed Proc *16* 165 166 1957

Crabtree H G Observations on the carbohydrate metabolism of tumours Biochem J 23 536 545 1929

Deschner F F & Gray I H Influence of oxygen tension on X ray induced chromo ... ascites tumor cells irradiated *in vitro* Radiation Res

... bolism of normal and tumour tissue IX Ammonia J 27 1123 1133 1933

... Metabolism of normal and tumour tissue XIV A medulla of kidney Bioch J *30* 659 660 1936

... der Korperzellen Biochem Ztschr *197* 175 188

Fujita ... 1928

FROM DEPARTMENT OF PATHOLOGY UNIVERSITY OF GOTHENBURG
SWEDEN (HEAD J MELLGREN MD)

EFFECT OF HEPARIN PROTAMINE, DICOUMAROL, STREPTOKINASE AND EPSILON AMINO N CAPROIC ACID ON THE GROWTH OF HUMAN CELLS IN VITRO[1]

By

ARNE LISSELL and JAN MELLGREN

Received 30 viii 62

A large number of tumor cells circulate in the blood of many cancer patients (31 38 40) Hence it is interesting to study factors capable of *influencing the tendency of tumor cells to settle down and develop into* metastatic growth

Wood (51) studied the devel pment f metastases *in vivo* He injected v tally stained ... an ear chamber ... to the vascular ... these cell Tl ... as es w th previous h stological observations in s ct ons f human and animal t ssue ind cat ng that the t mo ...

H wever ther bservati ns *ind cate* that heparin and d coumarol may exert a d rc t cyt stat r cyt t xic act on on b th ben gn and mal gnant *cells* Thus *F scher* (1) as well as *Bala s et al* (5 ...

F scher ... *their* ... cellular ... ...

This investigation was supported by research grants from *the Swedish Cancer Society*

Weinhouse S Millington R H & Wenner C E Metabolism of neoplastic tissue I The oxidation of carbohydrate and fatty acids in transplanted tumors Cancer Res *11* 845 850 1951
Weinhouse S Warburg O Burk D & Schade A L On respiratory impairment in cancer cells Science *124* 267 272 1956
Wood H G & Werkman C H The utilization of CO₂ by the propionic acid bacteria Bioch J *32* 1262 1271 1938
Yushok W D Metabolism of ascites tumor cells I Rate of glycolysis and competitive utilization of fructose mannose and glucose Cancer Res *19* 104 111 1959

F Merck AG Darmstadt, Germany) 2 g dissolved in 10000 ml of distilled water. The dispersions from three flasks were pooled and 120 ml of fluid medium added. 2 ml of the final cell suspension was added to each of about 60 roller tubes which were incubated immobile at 37° C. Every 24 hours the medium in all tubes was changed and the total protein content in three tubes from each series determined according to *Oyama & Eagle* (36). The average protein content in the three tubes was used as a measure of the cell quantity on a particular occasion. The average number of cells per tube after 24 hours' incubation was checked by counting in a Celloscope (AB Lars Ljungberg, Stockholm, Sweden) [illegible]

[illegible]

Sweden) and Epsilon amino n caproic acid (EACA) (Light & Co Ltd, Colnbrook, England)

[illegible]

[illegible] to the proteolytic enzyme fibrinolysin (plasmin), which *catalyzes* the decomposition of a series of proteins, among others haemoglobin and fibrin. Since the fluid medium contains human serum, streptokinase can act [illegible]

RESULTS

Heparin. The growths of the tested strains Bl II and C3 II, were *not* [illegible] % heparin [illegible]

[illegible] Six experiments were performed, four with Strain Bl II, two with Strain C3 II, and all the above mentioned concentrations of plasma *and heparin*. A typical experiment is illustrated in Fig. 2. After the third day, the cells regularly had similar growth rates in fluid medium alone and in fluid medium plus plasma, irrespective of the heparin concentration. However, addition of plasma to the fluid medium apparently gave a transient growth increment, less pronounced when heparin was *present*. *But*, because control experiments showed that the amount of protein adhering to the glass slightly increased when plasma—but not when plasma plus heparin—was added to mixtures of fluid medium and cell extract, it seems likely that this transient growth increment was *illusory* and actually due to precipitated fibrin.

Protamine. In three trials the growth of Strain Bl II, the only one tested, was not affected by 0.01 *mg%* protamine sulphate but distinctly

found that Strain L cells grown in suspension cultures containing 0 0001 0 1 per cent heparin showed no inhibition of mitosis or cell growth

On the other hand the view that heparin affects the cells directly is borne out by experiments in which heparin inhibited the growth of yeast cells (13) and bacterial division (43, 47) Moreover *Heilbrunn & Wilson* (18 19) reported that addition of heparin or dicoumarol to the medium prevented the premitotic gel state and thus mitosis itself from developing in fertilized Chaetopterus eggs *Lipman* (30) reported a clearly significant reduction of the mitotic index in and volume of Ehrlich's ascites tumor in mice after a single intraperitoneal heparin injection Moreover *Zakrzewski* (56) reported that Jensen sarcoma in rats and polymorphocellular sarcoma in mice showed reduced growth after heparin injections intravenously, intraperitoneally and into the tumor itself According to *Lacour et al* (28 29) orally administered dicoumarol exerted both growth inhibiting and metastasis reducing actions on homologous transplants of the rat uterine epithelioma T8 Guérin

Another mechanism whereby heparin could inhibit metastatic growth would be by reducing the permeability of the ground substance of vessels and connective tissue by virtue of its antihyaluronidase activity (10 14 16) *Balazs & Holmgren* (4 5) demonstrated that regeneration of injured tissue is retarded by the increase of its sulphomucopolysaccharide content *Prodi et al* (37) and *Ohlwiler* (34) reported that heparin inhibited wound healing Analogously *Jolles & Greening* (23) injecting heparin in and around sarcoma transplants in mice found that the growth of the transplants was retarded and the proportion of successful transplants lowered

Normally heparin is stored in connective tissue mast cells (2 24), apparently protecting the tissue against toxic lesions (20) Increased numbers of mast cells have been reported in the neighbourhood of animal tumors (3, 8 21) interpreted as a defensive reaction (8 21) perhaps through reduced tissue permeability *Koenig* (27) found that the number of mitoses was smaller the greater the number of mast cells in and around benzopyrine tumors in rats He concluded however that heparin from the mast cells directly inhibited tumor cell division

Lastly the literature suggests yet another metastasis inhibiting action of heparin *Agostino et al* (1) showed that heparin very quickly caused cancer cells to disappear from the systemic circulation Although the authors attributed the phenomenon to reduced fibrin formation or altered coagulability it seems more likely that the general haemodynamic effects of heparin were responsible Heparin is known to diminish erythrocytic aggregability (15) and in the light of *Fåhraeus* researches into the haemodynamic effects of erythrocyte aggregation upon blood flow it may be assumed that heparin causes the large malignant cells to travel in the axial current perhaps making them more liable to enter the smallest vessels where they might be destroyed or give rise to a large number of small or belatedly growing metastases Indeed *Zakrzewski* (56) found that heparin actually promoted the development of numerous but small metastases

Against this background, the aim of the present investigation was to ascertain whether some anticoagulants and fibrinolytics and their antagonists exert any direct cytostatic or cytotoxic action *in vitro* In order to eliminate the possibility of indirect growth inhibition by lysis of a coagulum substrate we decided to cultivate our strains direct on glass without a coagulum

MATERIALS AND METHODS

The test objects were three human cell strains established and maintained at this laboratory since March 1959 namely Strain B1 I emanating from non neoplastic bladder epithelium in a woman of 69 Strain B1 II emanating from a malignant bladder papilloma in the same patient and Strain C3 II established from a cervical carcinoma in a woman aged 36 The cultivation technique the fluid medium and the growth morphology and karyotype of the strains have been described previously (32 33) From 3 days cultures of these strains in flasks with 10 ml of fluid medium the medium was removed and the cells dispersed in 5 ml of this solution NaCl 80 g KCl 2 g Na_2HPO_4 11 5 g KH_2PO_4 2 g disodium versenate (Titriplex III

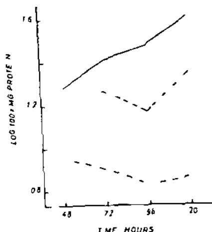


Fig 3

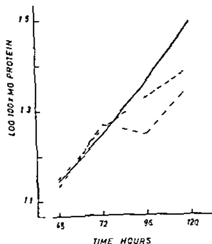


Fig 5

Fig 3 Effect f protamine on the gr wth of Strain Bl 11 *in vitro* The heavy con tinuous line (control) indicates the growth in fluid medium the dashed line the gr wth in flui l medium with 0 05 mg per cent protamine the dashed

Fig

d tte l line the growth in fluid medium with 20 mg per cent dicoumarol and the dotted line the growth in fluid med um with 100 mg per cent dicoumarol

mg% EACA respectively, in two experiments on Strain Bl 11 Since unlike the weaker concentrations of EACA 2500 mg% was markedly growth inhibiting the growth inhibiting effect of 2500 I U of streptokinase was not reduced One of the experiments is illustrated in Fig 7

None of the substances tested exerted any growth promoting action No difference in cellular morphology between the experimental series and the control series could be distinguished at microscopical examination

DISCUSSION

Thus in these experiments heparin did not exert a growth inhibiting effect even in high concentrations In this respect our findings differ from those previously reported These divergent results could be due to the use of entirely different cultivation techniques In our experiments the strains were cultivated directly on glass without a coagulum whilst other workers have all used a plasma coagulum Hence the previously reported growth inhibiting effect of heparin would seem to be due to its fibrinolytic destruction of the coagulum rather than to a direct mitotic inhibition

Apparently no previous studies have been published with respect to

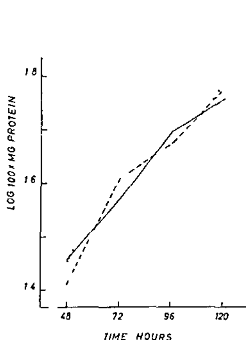


Fig 1

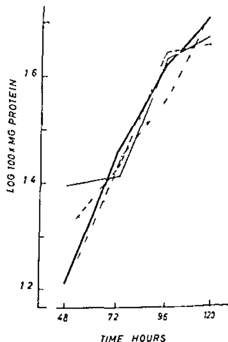


Fig 2

Fig 1 Effect of heparin on the growth of Strain C3 II *in vitro* The heavy continuous line (control) indicates the growth in fluid medium the dashed line in fluid medium with 1 mg per cent heparin and the dotted line the growth in fluid medium with 125 mg per cent heparin No influence on the growth by heparin can be demonstrated

Fig 2 Effect of human plasma and heparin on the growth of Strain Bl II *in vitr* The heavy continuous line (control) indicates the growth in fluid medium the thin continuous line the growth in fluid medium with 50 per cent human plasma the dashed line the growth in fluid medium with 50 per cent heparin plasma 0 05 per cent the dashed dotted line the growth in fluid medium with 50 per cent heparin plasma 0 1 per cent and the dotted line the growth in fluid medium with 50 per cent heparin plasma 0 5 per cent

inhibited by 0 05 and 0 1 mg% However, 0 25 mg% inhibited the growth only during the initial 24 hours, whereupon the growth again increased and ultimately became just as vigorous as that of the controls No change of pH of the medium could be detected during the course of the experiments One of the experiments is illustrated in Fig 3

Dicoumarol In three trials the growths of Strains Bl II and C3 II showed inhibitions proportional to the dicoumarol concentrations of 4, 20, and 100 mg%, as appears from Fig 4

Streptokinase The action of streptokinase was tested in five experiments on Strains Bl I, Bl II, and C3 II, their growths probably being inhibited by 500 and certainly by 2500 I U per ml fluid medium Fig 5

EACA The growths of Strains Bl I and Bl II were not convincingly inhibited in three experiments by 100 and 500 mg% of EACA, whilst 2500 mg% proved markedly growth inhibiting Fig 6

Streptokinase and EACA The growth inhibiting effect of up to 500 I U of streptokinase per ml fluid medium was reduced by 100 and 500

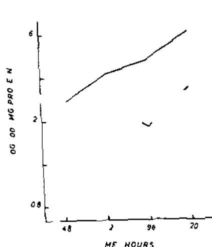


Fig 3

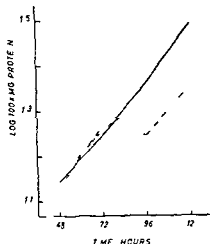


Fig 4

Fig 3 Effect f protam ne n the gr wth f Stra n Bl II n i tr The heavy c n t nu us l ne (contr l) ind cates the gr th n fluid med um the dashed l ne the g owth in fluid med um with 0 05 mg per cent protam ne the dashed dotted l ne the gr th in flu d med um with 0 1 mg per cent pr tam ne and the dotte l line the growth in fluid med um w th 0 25 mg per cent pr tam ne

Fig 4 Effect of d cou marol on the gr wth of Strain Bl II in v tro The heavy c n tinuous l ne (control) ind cates the growth in flui l me lium the dashed l ne the growth in flu d med um w th 4 mg per cent d coumarol the dashed d tted l ne the gr th in flui l me l um w th 20 mg p r cent d coumarol and the dotted l ne the growth in flu d me lium with 100 mg per cent dicoumar l

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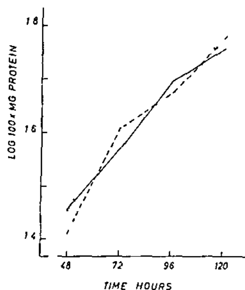


Fig 1

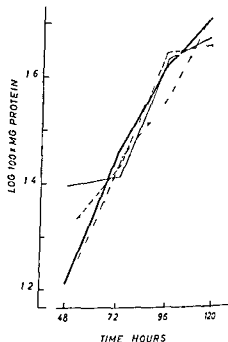


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LOG 100× MG PROTEIN

TIME HOURS

Fig 7

Effect of streptokinase and EACA on the growth of Strain Bl 1I *in vitro* The heavy continuous line (control) indicates the growth in fluid medium the dashed line the growth with 100 I U streptokinase per ml fluid medium and 100 mg per cent EACA the dashed dotted line the growth with 500 I U of streptokinase per ml fluid medium and 500 mg per cent EACA and the dotted line the growth with 2500 I U of streptokinase per ml fluid medium and 2500 mg per cent EACA

SUMMARY

A review of the effects of coagulation inhibitors on tumor cell growth and on metastasis seems to indicate different possible mechanisms, among others a direct cytotoxic effect

Human cell strains grown directly on glass in fluid medium containing 1 to 125 mg% heparin showed no inhibition of cell growth Protamine, dicoumarol, streptokinase, and EACA all inhibited the growth with rising concentrations

REFERENCES

1 [illegible] rin on Circulating [illegible] 17 1961
2 [illegible] 1954
3 [illegible] ntercellular M[illegible]
4 Bala [illegible]
5 Balc [illegible] The Biological Activity of Hyaluron Sulfuric Acid Acta Physiol Scand 23 168 1951
6 Cliffton E E & Agostino D Factors Affecting the Development of Metastatic Cancer Cancer 15 276 1962
7 Cliffton E F & Grossi C E Effect of Human Plasmin on the Toxic Effects and Growth of Blood Borne Metastasis of the Brown Pearce Carcinoma [illegible]
8 Cr[illegible]
9 De[illegible]

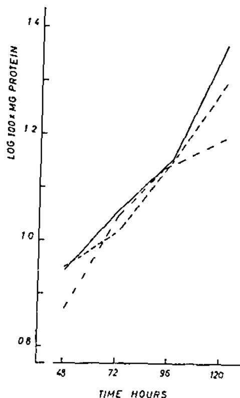


Fig 5

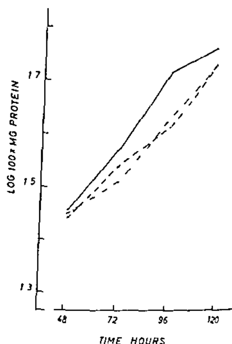


Fig 6

Fig 5 Effect of streptokinase on the growth of Strain Bl II *in vitro* The heavy continuous line (control) indicates the growth in fluid medium the dashed line the growth with 100 I U streptokinase per ml fluid medium the dashed dotted line the growth with 500 I U streptokinase per ml fluid medium and the dotted line the growth with 2500 I U of streptokinase per ml fluid medium

Fig 6 Effect of epsilon-amino n caproic acid (EACA) on the growth of Strain Bl II *in vitro* The heavy continuous line (control) indicates the growth in fluid medium the dashed line the growth in fluid medium with 100 mg per cent EACA the dashed dotted line the growth in fluid medium with 500 mg per cent EACA and the dotted line the growth in fluid medium with 2500 mg per cent EACA

the effect on tissue cultures of dicoumarol, protamine, streptokinase, and EACA The results of the present investigation indicate that with rising concentrations they are all growth inhibiting, even though the behaviour of protamine is somewhat confusing The effect of streptokinase would seem to be to activate the fibrinolytic system with consequent proteolytic action on the cells Such an assumption is borne out by the opposite effect of EACA

The results of these experiments suggest that the metastasis inhibiting action of heparin should not be attributed to a direct cytotoxic action but rather to one of the other mechanisms of action discussed in the introduction Although dicoumarol and fibrinolysin at high concentrations inhibit the growth *in vitro* it is uncertain whether this effect is of any importance in their metastasis inhibiting properties *in vivo*

LOG 100× MG PROTEIN

TIME HOURS

Fig 7

Effect of streptokinase and EACA on the growth of Strain BI H *in vitro* The heavy continuous line (control) indicates the growth in fluid medium, the dashed line the growth with 100 I U streptokinase per ml fluid medium and 100 mg per cent EACA the dashed dotted line the growth with 500 I U of streptokinase per ml fluid medium and 500 mg per cent EACA and the dotted line the growth with 2500 I U of streptokinase per ml fluid medium and 2500 mg per cent EACA

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A review of the effects of coagulation inhibitors on tumor cell growth and on metastasis seems to indicate different possible mechanisms, among others a direct cytotoxic effect

Human cell strains grown directly on glass in fluid medium containing 1 to 125 mg% heparin showed no inhibition of cell growth Protamine, dicoumarol, streptokinase, and EACA all inhibited the growth with rising concentrations

REFERENCES

1 Agostino D [illegible] Circulating [illegible] 961
2 A [illegible]
3 A [illegible] llular Muco [illegible]
4 Bala [illegible]
5 Bala [illegible] 1951
6 *Clifton E E & Agostino D* Factors Affecting the Development of Metastatic Cancer Cancer *15* 276 1962
7 *Clifton E E & Grossi C E* Effect of Human Plasmin on the Toxic Effects and Growth of Blood Borne Metastasis of the Brown Pearce Carcinoma and the V2 Carcinoma of Rabbit Cancer 9 1147 1956
8 *Cramer W & Simpson W L* Mast Cells in Experimental Skin Carcinogenesis Cancer Res *4* 601 1944
9 *Deutsch F* Blutgerinnungsfaktoren Frank Deuticke Wien 1952

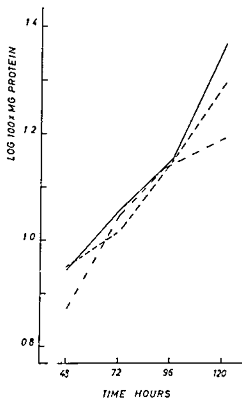


Fig 5

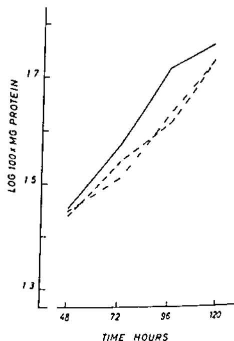


Fig 6

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Fig 6 Effect of epsilon-amino n caproic acid (EACA) on the growth of Strain Bl II *in vitro* The heavy continuous line (control) indicates the growth in fluid medium the dashed line the growth in fluid medium with 100 mg per cent EACA the dashed dotted line the growth in fluid medium with 500 mg per cent EACA and the dotted line the growth in fluid medium with 2500 mg per cent EACA

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38 *Roberts S Waine A McGrath R McGrew E A & Cole, W H* Technique and Results of Isolation of Cancer Cells from the Circulating Blood A M A Arch Surg *76* 334 1958
39 *Rosenlund B & Foss O P* Automatic Counting of White Blood Cells Nord Med *63* 556 1960
40 *Sandberg A A Moore C E, Crosswhite L H & Schubarg J R* Frequency of Tumor Cells in Bone Marrow and Blood Cancer *11* 1180 1958
41 *Saphir O* The Fate of Carcinoma Emboli in the Lung Am J Path *23* 245, 1947
42 *Schmidt M B* Die Verbreitungswege der Karzinome und die Beziehung generalisierter Sarkome zu den leukämischen Neubildungen Gustav Fischer Jena 1903
43 *Stoker S B* Bacteriostatic Action of Heparin J Physiol, *110* 26 1949
44 *Takahashi M* An Experimental Study of Metastasis J Path & Bact, 20 1 1915
45 *Terranova T & Chissone F* Il Fattore Coagulazione nell'Attecchimeto delle Cellule Neoplastiche immesse in Circolo Boll Soc ital Biol sper, *28* 1224 1952
46 *Walther H E* Krebsmetastasen Benno Schwabe & Co, Basel 1948
47 *Warren S & Gates O* The Fate of Intravenously Injected Tumor Cells Am J Cancer *27* 485 1936
48 *Warren J R & Graham F* The Effect of Heparin on the Growth of Bacteria and Yeasts J Bact *60* 171 1950
49 *Willis R A* Spread of Tumors in the Human Body 2nd ed The S V Mosby Co St Louis 1952
50 *Winterstein A* Chemie der Antik [illegible] Hamburger Symposium 2 13 Apr [illegible]
51 *Wood S Jr* Pathogenesis of Me[illegible] n the Rabbit Ear Chamber A M A [illegible]
52 *Wood S Jr Holyoke F D & Yardley J H* An Experimental Study of the Influence of Adrenal Steroid Growth Hormone and Anticoagulants on Pulmonary Metastasis Formation in Mice Proc Am A Cancer Res 2 157 1956
53 *Wood S Yardley J H & Holyoke F D* The Relationship Between Intravascular Coagulation and the Formation of Pulmonary Metastases in Mice Injected Intravenously with Tumor Suspension Proc Am A Cancer Res 2 260 1957
54 *Zakrzewski Z* Untersuchungen über den [illegible] tum der Sarkomzellen [illegible]
55 *Zakrzewski Z* Die Rolle d[illegible]
56 [illegible] Wachs- Pol Sci [illegible] 1932

10 *Duran Reynals F* Tissue Permeability and Spreading Factors in Infection Contribution to Host Parasite Problem Bact Rev *6* 197 1942

11 *Fischer A* Ueber die Wirkung des Heparins auf das Wachstum von Gewebe zellen in Vitro Protoplasma *26* 344 1936

12 *Fischer A* Die Bindung von Heparin an lebende Zellen Scand Arch Physiol 75 121 1936

13 *Fischer A & Nystrom P* Heparin als Wachstumshemmender Stoff Biochem Ztg 262 364 1933

14 *Fritze E* Cellulare und Humorale Abwehr Systeme und Reaktionen Ergebn Inn Med Kinderheilk *9* 322 1958

15 *Fåhraeus R* Personal communication

16 *Glick D & Sylven B* Evidence for Heparin Nature of Nonspecific Hyaluronidase Inhibitor on Tissue Extracts and Blood Serum Science (Lancaster Pa) *113* 388 1951

17 *Grossi C E Agostino D & Clifton E E* The Effect of Human Fibrinolysin on Pulmonary Metastases of Walker 256 Carcinosarcoma Cancer Res *20* 605 1960

18 *Heilbrunn L V* The Dynamics of Living Protoplasm Academic Press Inc New York 1956

19 *Heilbrunn L V & Wilson W L* The Effect of Heparin on Cell Division Proc Soc Exp Biol & Med *70* 179 1949

20 *Higginbotham R D & Dougherty T F* Heparin a Biologic Ion Exchanger in Protection vs Noxius Agents Fed Proc *16* 58 1957

21 *Holmgren H & Wohlfart G* Mast Cells in Experimental Rat Sarcomas Cancer Res 7 686 1947

22 *Iwasaki T* Histological and Experimental Observations on the Destruction of Tumor Cells in the Blood Vessels J Path & Bact *20* 85 1915 16

23 *Jolles B & Greening S G* Effect of Heparin upon Tumor Growth Unio Inter nat Contra Cancrum Acta *16* 682 1960

24 *Jorpes J E* Heparin Its Chemistry Physiology and Application in Medicine Oxford Univ Press London 1939

25 *Kaulla K N von McDonald S T & Taylor G H* The Effect of Heparin on Fibrinolysis J Lab Clin Med *48* 952 1956

26 *King D W Bensch K G & Simbonis S* The Lack of Effect of Heparin on Mitosis in Strain L Cells Cancer Res *18* 382 1958

27 *Koenig W* Ueber die Bedeutung von Mastzellen im Benzpyren Tumor von Ratten Z f Gesamte Exp Med *120* 64 1955

28 *Lacour F Oberling D & Guerin M* Influence de l'ethyldicoumarol sur l'evolution des metastases de l'épithelioma T8 chez le rat Note préliminaire Bull Assoc franç étude cancer *42* 531 1955

29 *Lacour F Oberling C & Guerin M* Influence de l'ethyldicoumarol sur l'evolution des métastases de l'epithelioma T8 chez le rat Nouvelles recherches Bull Assoc franç etude cancer *43* 44 88 1956 57

30 *Lippman M S* The Growth Inhibitory Action of Heparin on the Ehrlich Ascites Tumor in Mice Cancer Res *17* 11 1957

31 *Malmgren R A Pruitt J C Del Vecchio P R & Potter J F* Method for Cytologic Detection of Tumor Cells in Whole Blood J Nat Ca Inst *20* 1203 1958

32 *Mellgren J Boeryd B & Hagman M* Growth Potentials of Precancer of the Cervix Uteri in Vitro and in Cortisone Treated Hamsters Cancer Res 2? 139 1962

33 [illegible] *O & Mellgren J* Growth Morphology and Karyotype in [illegible] and Benign Origin from the [illegible]

34 [illegible] Acid upon the Formation [illegible] Vol X 1959

35 *Ouu* [illegible] Resistance Nord Med *62* 1421 1959

36 *Oyama V I & Eagle H* The Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin Ciocalteau) Proc Soc Exp Biol & Med *91* (2) 305 1956

37 *Prodi G Baggi G & Tassi T* Effect of Heparin on Cutaneous Lesions Produced by Staphylococci Boll Soc Ital Biol sper *33* 514 1957

the edge of a ring For sectioning in other planes sectors of the embedded rings were cut out and re embedded in methachrylate in the plane of sectioning desired Sections were cut on a LKB ultramicrotome and studied in a Philips EM 100 B

OBSERVATIONS

None of the aortae from which specimens were obtained presented gross pathological changes Neither did careful phase microscope studies reveal any abnormalities in the sections cut for orientation purposes from all specimens used

As far as general features are concerned the present study has added only little to the classical concept of the structure of the aortic wall The endothelial cells of the tunica intima form a continuous lining of the luminal surface of the wall A subendothelial layer connects the endothelium with the innermost elastic lamella of the tunica media The tunica media forms an elastic tube around the tunica intima and is composed of alternating coaxial layers of elastic lamellae and smooth muscle cells The disposition of the layers is seen in Fig 1 In all between 24 and 27 ramifying elastic lamellae of appreciable thickness are found in the tunica media of the rabbit aorta Smooth muscle cells are the only cells to be found in the tunica media They extend more or less diagonally across interlamellar spaces, binding together adjacent elastic lamellae As a third component of the tunica media loosely-packed collagen fibers or bundles of fibers fill the interlamellar spaces where these are not occupied by smooth muscle cells or branches of elastic tissue

The following is a description of new structual information obtained with the electron microscope

Tunica Intima

Endothelium The appearance of the endothelial cells (Figs 2 to 5) was found to be essentially that reported by *Buck* (1958) The cells closely resemble the endothelial cells of the rat aorta (*Keech* 1960, Pease & Paule 1960) Further, their internal structure is in accordance with that of endothelial cells of capillaries and small arteries (*Palade* 1953 *Moore & Ruska* 1957), of coronary arteries (*Parker* 1958) and of the atrium of the turtle heart (*Fawcett & Selby* 1958) It is worthy of note however, that the aortic endothelium differs from the endothelium of smaller arteries and capillaries in not being applied to a basement membrane This morphological feature, probably implying a high permeability of the endothelium, may be tentatively connected with the absence of vasa vasorum in the tunica media of the aorta

The endothelial cells are short and more or less protruding into the lumen, their protrusion as well as their length possibly varying with the degree of stretching or contraction of the wall They join by contact of their plasma membranes which occasionally form interdigitating

LABORATORY OF ELECTRON MICROSCOPY AND CONNECTIVE TISSUE RESEARCH LABORATORY, MEDICINSK-ANATOMISK INSTITUTE UNIVERSITY OF COPENHAGEN

ELECTRON MICROSCOPY OF THE NORMAL RABBIT AORTA

By

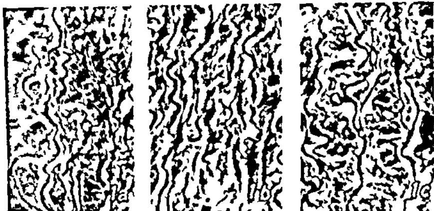
F BIERRING and T KOBAYASI

Received 19 ix 62

Studies on the ultrastructure of the normal mammalian aorta so far have been confined largely to the rat and the mouse (*Berrian* 1953, *Keech* 1960, *Pease & Paule* 1960, *Karrer* 1961) Reports of studies on the normal rabbit aorta have been concerned only with certain aspects of the ultrastructure of its component parts *Buck* (1958) described the pinocytotic vesicles in the endothelial cells as a possible mechanism in the transport of fluid through the aortic endothelium, and *Takamatsu* (1958) studied the fine structure of the elastic elements of the tunica media The present study deals with the morphology of the tunicae intima and media of the rabbit thoracic aorta It was designed to provide the basis for electron microscope studies on the changes occurring in epinephrine-induced arteriosclerosis of the rabbit aortic wall The results of these studies will be reported in a separate paper

MATERIAL AND METHODS

Young white rabbits weighing approximately 1 5 kg were used in the investigations Under nembutal anaesthesia and artificial respiration the left half of the
thoracic aorta exposed Segments of the aorta
solution in veronal acetate buffer (pH about
—in 10 per cent formalin In all cases of OsO_4
fixation the fixation was commenced *in situ* Here the most satisfactory results were obtained after adoption—with only insignificant modifications—of the technique described by *Pease & Paule* (1960) for the fixation of the rat thoracic aorta With this method a segment of the aorta is preserved in a state of approximately normal expansion The total period of OsO_4 fixation was 90 120 minutes The specimens were dehydrated in graded concentrations of ethanol beginning with 20 per cent During the dehydration the adventitia was carefully removed by dissection in order to facilitate subsequent sectioning Further at this stage the aortic segment was cut into rings 1 to 2 mm in height All specimens were stained by adding 1 per cent of phosphotungstic acid to the second of the three final changes of absolute alcohol
the specimens were kept in this solution for a period of 40 minutes The speci
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and it was hoped in this way
distortion of the structures of the wall Transverse sections of the wall were cut from



folds Along the cell junctions localized areas of slightly increased density of the plasma membranes and of the adjoining cytoplasm (Fig 5) suggest the presence of poorly defined attachment zones Localized dilatations of the intercellular spaces may be found

The cytoplasm of the endothelial cells exhibits a finely filamentous texture (Fig 5) with minute filaments randomly disposed in the cytoplasmic matrix Small plasma membrane invaginations and small vesicles line the luminal and basal cell surfaces as well as the surfaces along the intercellular boundaries Small smooth-surfaced vesicles and sometimes larger vesicles are also found well inside the cells They correspond to the pinocytotic vesicles of the endothelium of smaller blood vessels, described by *Palade* (1953) and by *Moore & Ruska* (1957) as a hypothetical mechanism of the transcellular transport of fluid Apart from vesicles a few small, typically dense, mitochondria, a Golgi complex and sparse elements ascribable to an endoplasmic reticulum are found Further, inclusions in the form of osmiophilic small dense bodies (Fig 2) are irregularly scattered in the cytoplasm They resemble the dense bodies found in the aortic endothelium of the rat by *Pease & Paule* (1960) and of the rabbit by *Buck* (1958), who demonstrated them to be inclusions concerned with phagocytosis

Subendothelial layer The endothelium is supported by a subendothelial layer of appreciable width It is defined as the layer which extends from the basal plasma membrane of the endothelium to the luminal aspect of the first thick sheet of elastic tissue (Figs 3 and 5) This sheet correspond to the innermost elastic lamella of the tunica media of light microscopy

The subendothelial layer is made up of branching elastic fibers and sparse collagen fibers embedded in a homogeneous ground substance of low density The low density of the ground substance is probably an extraction effect resulting from a high water content Spindle shaped cells (Figs 3 and 4) resembling smooth muscle cells are occasionally observed in the deep part of the layer close to the innermost elastic lamella Most conspicuous are the branching elastic fibers, some of which are continuous with the innermost elastic lamella (Figs 2 and 5) The elastic fibers form a more or less well-pronounced fenestrated

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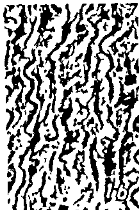
Figs 1 2

Fig 1 Phase contrast photomicrographs of transverse sections through the tunica intima and inner part of the tunica media (a) middle (b) and outer (c) parts of the tunica media of a normal rabbit aorta

Fig 2 Electron micrograph of a transverse section through the wall of a normal rabbit aorta showing endothelial cells (E) subendothelial layer first (ei) second and third elastic lamellae of the tunica media and the smooth muscle cells of the two inner interlamellar spaces In the second interlamellar space

In the subjacent first interlamel
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lane muscle cells are obliquely
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membrane (Figs 3 and 4) which lies approximately parallel to the basal endothelial surface dividing the subendothelial layer into a narrow luminal and a wider deep part The fenestrated membrane is also visible in the phase contrast micrograph of Fig 1 a

The collagen fibers of the subendothelial layer are found mainly in the deepest part lying in bundles close to the innermost elastic lamella Fig 5) intermingled with branching elastic fibers They are of moderate size (approximately 400 A in diameter) In transverse sections of the wall they are mostly seen in cross section Sections cut at a different angle reveal their cross banding

Few collagen fibers are found on the luminal side of the fenestrated elastic membrane This part of the subendothelial layer is however permeated by small fragments of elastic tissue (Fig 5) In places the fragments are seen to pass into the fenestrated elastic membrane and their dimensions decrease as they approach the endothelium They may be taken thus to represent fine branches from the fenestrated elastic membrane They are surrounded by dense delicate filaments (see also Fig 7) As will be considered later the delicate filaments possibly are elastic unit fibers and in the following this term will be used to describe them

The fine elastic branches approach the basal plasma membrane of the endothelium in an oblique tangential manner (Fig 5) Their orientation towards the endothelium is perhaps best shown in Fig 3 Here the subendothelial layer has an appearance which is suggestive of swelling during fixation procedures and elastic branches are seen to take an almost radial course towards the endothelium

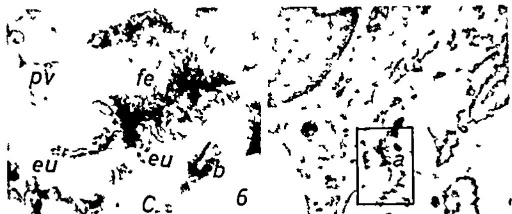
In areas where elastic elements reach the endothelium the basal endothelial plasma membrane and the subjacent cytoplasm display a special structural pattern which is slightly reminiscent of that described by *Selby* (1955) and *Odland* (1958) in the basal epidermal cells as possibly related to the attachment of epidermal cells to the dermis At areas of contact with elastic elements the plasma membrane is slightly thickened Endothelial cytoplasmic filaments converge towards and are densely packed in the cytoplasm immediately subjacent to the thickening of the plasma membrane At lower magnifications (Figs 3 4 and 5) one or two dense areas may be seen on the basal surface of each cell An example of a dense area is seen at a high magnification in Fig 6 The regular occurrence of dense areas along the endothelial plasma

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Figs 3 4

Fig 3 The subendothelial layer of this section has an appearance suggesting swelling during preparation The elastic fibers of its luminal part are radially oriented towards the endothelium Note the dense area at the apex of a cytoplasmic process (a) which is apparently anchored by elastic fibers

Fig 4 Endothelium and subendothelial la[illegible]





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membrane makes it unlikely that they simply represent fortuitous relations between elastic fibers and the plasma membrane. The impression, however, that they represent zones of attachment serving to anchor the endothelium is especially borne out by findings like that presented in Fig. 3. A radially oriented row of elastic elements is continuous peripherally with a dense area of an endothelial cell. The apparently edematous ground substance seems to bulge into the endothelial cell which is flattened in its peripheral parts. In this case the dense area is found at the apex of a cytoplasmic process, apparently retaining its position during the swelling of the ground substance as a result of being anchored by elastic fibers.

Collagen fibers have never been observed in close association with the endothelial plasma membrane.

Tunica Media

The observations of the present study agree on all main points with the observations on the aortic tunica media of other mammals reported in the previously cited papers by *Keech* (1960), *Pease & Paule* (1960) and *Karrer* (1961) and will only be summarized. Additional observations supplementing those already available will be described in more detail. Special attention has been paid to the fine structure of the elastic tissue in the tunica media.

Fine structure of elastic tissue. In the phosphotungstic acid-stained preparations elastic lamellae and their branches have an apparently homogeneous interior of moderate electron density and an outer thin, frayed coating of relatively dense material (Figs. 8, 10, 11, 14 and 15). Occasionally, however, an additional component is found in the otherwise uniform interior. This component can be seen in Figs. 9, 12 and 13, where it appears as fairly regularly spaced, thin streaks of dense material oriented along the long axis of an elastic fiber. In cross-sections the streaks appear as dots. They are seen more clearly after fixation in formalin (Figs. 12 and 13) than after fixation in OsO_4. The presence of streaks of dense material embedded in the vascular elastin has been

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Figs. 5–8

Fig. 5. [illegible]

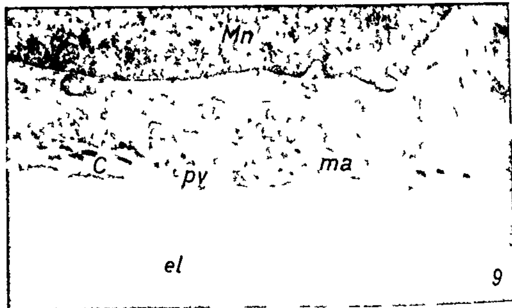
Fig. [illegible]

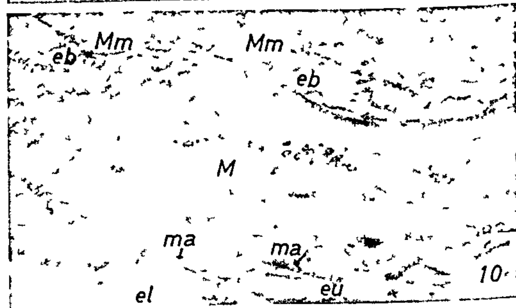
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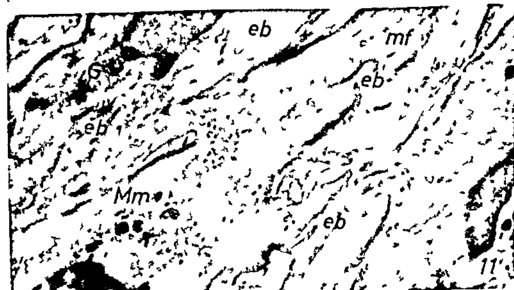
in [illegible] × 72 500

Fig. 7. Elastic fragments of transversely sectioned fine elastic branches from the subendothelial layer surrounded by elastic unit fibers (eu). × 72 500

Fig. 8. Muscle cell attachments to elastic lamella. The cytoplasm along the plasma membrane which borders the elastin shows areas of increased density. Elastic unit fibers (eu) protrude from the surface of the elastic lamella. × 26 700


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reported by *Parker* (1958), *Takamatsu* (1958), *Keech* (1960) and by *Karrer* (1961), who also points out that the streaks are only occasionally seen within the elastin Micrographs like that shown in Fig 12 give the impression that elastic fibers are made up of bundles of fibrils held together by the dense material appearing as streaks The idea receives support from the fact that branching of the fibers occur along a line of dense material (Fig 12)

Delicate filaments with a diameter of about 50–100 Å emanate from the surface of elastic fibers (Figs 8, 10, 11 and 15), and elastic fibrils occasionally appear to end in tufts of similar filaments The filaments are the finest elastic elements seen in the electron micrographs They appear uniformly dense and they do not divide further These facts seem to justify the conclusion that they represent elastic unit fibers The term is used in order to express the tentative point of view that they are structural units which by aggregation, possibly with the aid of an interfilamentous substance form fibrils which are again organized into fibers

The dense outer coating of the elastic fibers is probably accounted for by the fringe of minute filaments issuing from their surfaces Evidence to support this conjecture is shown to advantage in Figs 4, 8, 9 10 and 11

Histological features of the tunica media The elastic lamellae appear as practically continuous sheets of appreciable thickness with only occasional gaps However, the innermost 2 to 3 lamellae in places are discontinuous (Fig 2) In this part of the tunica media interlamellar spaces frequently communicate through large fenestrae in the lamellae *Elastic fibers* form a coarse network in the interlamellar spaces and presumably represent branches of the elastic lamellae as connections between fibers and lamellae are sometimes observed

Elongated smooth muscle cells span the interlamellar spaces, extending from one lamella to the next They are attached to the *elastic lamellae* and their branches, binding together adjacent lamellae The electron micrographs presented here (*e g Fig 2*) merely suggest their spatial orientation but appropriate sections reveal that they run obliquely to both the radial and the *longitudinal* axes of the aorta Their angles to the long axis probably vary from one space to the next Wider inter

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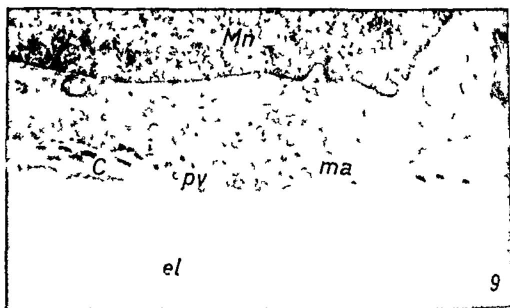
Figs 9 11

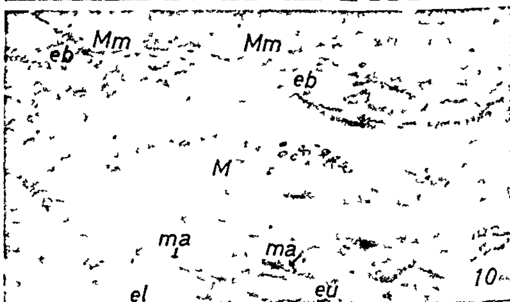
Fig 9 An area of muscle cell attachment (ma) to the surface of an elastic lamella (el) A narrow space is seen between the plasma membrane and the elastic lamella. × 26 700

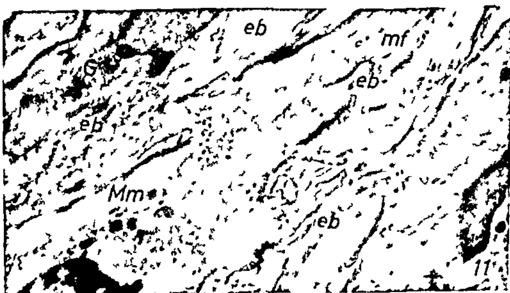
Fig 10 D

Fig 11 [illegible] sectioned muscle cells An apparent intercellular bridge connects the two uppermost cells × 12 400

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Figs 9 11

Fig 9 An area of muscle cell attachment (ma) to the surface of an el...

Fig 10

...ated × 20 400

Fig 11 Portions of longitudinally sectioned muscle cells An apparent intercellular bridge connects the two uppermost cells × 12 400

lamellar spaces separate the outermost 2 to 3 elastic lamellae and the muscle cells of these spaces are radially oriented (Fig 1 c)

Although their fine structure has not been investigated in detail this study has confirmed that the morphology of the aortic muscle cells differs in certain respects from that of smooth muscle cells from other sites (*Pease & Paule* 1960, *Karrer* 1961) They are peculiar in the rather poor development of their myofilaments which are longitudinally arranged and parallel (Fig 14) The cells are separated from each other by interspaces containing collagen fibers and occasionally elastic fibers (Figs 11 and 14), yet areas may be found where muscle cells are closely apposed with their plasma membranes separated only by a narrow space of uniform density (Figs 4 and 11) Apparent cytoplasmic anastomoses between muscle cells are found on rare occasions (Fig 11) recalling the observations of *Mark* (1956) and *Thaemert* (1959) on uterine and gastrointestinal smooth muscle cells The existence of true anastomosing bridges between smooth muscle cells has been rejected by *Caesar, Edwards & Ruska* (1957) The question of their existence between aortic muscle cells cannot be answered unequivocally although the appearance of the "intercellular bridge" in Fig 10 suggests that they must be interpreted with caution A thin but distinct basement membrane, separated from the plasma membrane by a narrow space, is visible at the lateral cell borders, except where muscle cells are closely apposed In such areas absence of basement membranes permits an intimate apposition of plasma membranes Pinocytotic vesicles generally are restricted to the cytoplasm immediately beneath the plasma membrane

The muscle cell attachments to elastic lamellae or their branches (Figs 8, 9 10, 11 and 14) display a structure which is consistent with that described in the rat aorta In the attachment zones the muscle cell cytoplasm bordering the plasma membrane is usually of an increased density A narrow space of low, yet slightly positive density separates the plasma membrane from the surface of the elastin The space is comparable to that underlying the basement membrane of the lateral cell borders It has not been possible in the sections obtained to deter-

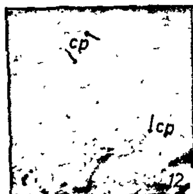
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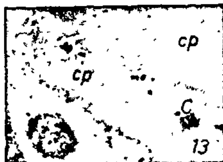
Figs 12 15

Figs 12 and 13 Portions of elastic lamellae Fairly regularly spaced thin streaks of dense material (ep oriented along the long axis are seen in the interior of the elastic tissue The elastic lamella of Fig 12 seems to branch along a line of dense material (Formalin fixed and stained with phosphotungstic acid prior to embedding) × 20 600

Fig 14 Portions of two muscle cells separated by a wide interspace containing elastic branches and collagen fibers Longitudinally arranged parallel myofilaments are seen in the cell to the left × 6 300

Fig 15 High power micrograph of elastic fibers which display an apparently homogenous interior Delicate filaments which may be interpreted as elastic unit fibers emanate from their surfaces (OsO_4 fixed and stained with phosphotungstic acid prior to embedding) × 72 500


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mine with certainty whether this space is crossed by elastic unit fibers. Where the elastic lamellae are not in contact with muscle cells they are invested by mainly longitudinal bundles of collagen fibers which also fill the spaces between muscle cells and occasionally intervene between muscle cells and elastic lamellae (Fig. 9). Collagen fibers may lodge in indentations of a lamellar surface (Figs. 3 and 5) probably explaining the occasional findings of collagen fibers which are apparently completely surrounded by elastin (Fig. 13).

It is to be emphasized, finally, that neither blood vessels nor nerve fibers have been observed in any part of the tunica media.

DISCUSSION

Previous accounts on electron microscopy of mammalian aorta disagree to some extent in the relationship between endothelium and tunica media and especially in the interpretation of the occasional occurrence of elastic elements between the two layers. *Karrer* (1961), studying the aorta of young and aging mice, observed that a subendothelial layer containing elastic elements typically occurred only in older aortas, and he proposed that its appearance indicated the rarification and fragmentation of elastin which he generally found in aging aortas. In the rat, *Pease & Paule* (1960) found that in places aortic endothelium was directly attached to the internal elastic lamella but elsewhere was separated from it by a layer containing elastic fragments. The latter authors interpreted these fragments as centers of elastin deposition.

Evidence from the present study supports the idea advanced by *Karrer* (1961) of a subendothelial layer arising through aging changes. The fenestrated elastic membrane, the large fenestrae in the inner elastic lamellae and the occasional occurrence of cells resembling smooth muscle cells all tend to show that the existence in the adult rabbit aorta of a wide subendothelial layer probably results from a degenerative transformation of the inner parts of the tunica media. The findings here presented are on the other hand incompatible with the alternative hypothesis of elastin formation in the subendothelial layer. Endothelial cytoplasm admittedly contains minute filaments which are almost of the same size and density as the elastic unit fibers and this similarity might suggest an initial phase of elastogenesis in these cells. The sparse occurrence in the cells of structures normally associated with synthetic processes however is evidence against such an interpretation.

In spite of the presence of a subendothelial layer endothelial cells apparently – via elastic fibers – maintain their attachments to the elastic elements of the tunica media. Reasons for regarding the areas where elastic fibers are in contact with the plasma membrane as zones of endothelial attachment have been given already. As, however, the existence of these zones represents a novel structural feature a few

points related to their structure merit further discussion An alternative interpretation of the supposed attachment zones is the possibility that the apparently cytoplasmic filaments of the condensed area beneath the plasma membrane are in reality extracellular elastic unit fibers which at an angle of section oblique to the cell surface are *projected* on to the cytoplasmic area This for instance might be the case in the left part of the area shown in Fig 6 In the right part of the area however this *can hardly be the case as the plasma membrane* can be followed as a distinct line showing that the section has been cut almost perpendicular to its surface In practically all instances studied a plasma membrane of an even increased density intervened between elastic and cytoplasmic filaments This observation together with those previously mentioned seems to confirm that the dense areas represent more than accidental topographical relationships *Kobayasi* (1961) studying the *dermo epi*dermal junction found delicate filaments probably of elastic tissue in the space between the corium membrane and the plasma membrane of the basal epidermal cells This observation suggests a certain similarity between zones of epidermal and of endothelial attachments

The finding of endothelial cell attachments to elastic tissue encourages the obvious thought about their significance in pathological changes of aging aortas in which a rarification of elastic tissue is known to *occur* Our present knowledge permits only conjecture at this point

Key to abbreviations used in the electron micrographs

E	Endothelial cell	pv	Pinocytotic vesicles
el	Elastic lamella	F	Fenestra
ei	Innermost elastic lamella of tunica media	*Mn*	*Muscle cell nucleus*
eb	Elastic branch	*mf*	Myofilaments
eu	Elastic unit fibers	*ma*	Attachment of muscle cell to elastic tissue
M	Smooth muscle cell	*Mm*	Area of contact between muscle cells
C	Collagen fibers	*G*	Golgi complex
ep	Filamentous structure in elastic tissue	a	Attachment of endothelial cell
fe	Filaments in endothelial cytoplasm	*d*	dense bodies in endothelial cells

SUMMARY

Morphological features of the aortic tunica intima and media of young adult rabbits have been investigated as a basis for electron microscope studies on *epinephrin*-induced arteriosclerosis A subendothelial layer of appreciable width *containing* is its most conspicuous element numerous branching elastic *fibers separates* the endothelium from the tunica media The morphological evidence [illegible]

[illegible] basal endothelial plasma membrane represent zones where *endothelial* cells are attached to elastic elements of the subendothelial layer Thin streaks of dense material occasionally

mine with certainty whether this space is crossed by elastic unit fibers. Where the elastic lamellae are not in contact with muscle cells they are invested by mainly longitudinal bundles of collagen fibers which also fill the spaces between muscle cells and occasionally intervene between muscle cells and elastic lamellae (Fig. 9). Collagen fibers may lodge in indentations of a lamellar surface (Figs. 3 and 5) probably explaining the occasional findings of collagen fibers which are apparently completely surrounded by elastin (Fig. 13).

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Evidence from the present study supports the idea advanced by *Karrer* (1961) of a subendothelial layer arising through aging changes. The fenestrated elastic membrane, the large *fenestrae in the inner elastic* lamellae and the occasional occurrence of cells resembling smooth muscle cells all tend to show that the existence in the adult rabbit aorta of a wide subendothelial layer probably results from a degenerative transformation of the inner parts of the tunica media. The findings here presented are on the other hand incompatible with the alternative hypothesis of elastin formation in the subendothelial layer. Endothelial cytoplasm admittedly contains minute filaments which are almost of the same size and density as the elastic unit fibers and this similarity might suggest an initial phase of elastogenesis in these cells. The sparse occurrence in the cells of structures normally associated with synthetic processes however is evidence against such an interpretation.

In spite of the presence of a subendothelial layer, endothelial cells apparently – via elastic fibers – maintain their attachments to the elastic elements of the tunica media. Reasons for regarding the areas where elastic fibers are in contact with the plasma membrane as zones of endothelial attachment have been given already. As, however, the existence of these zones represents a novel structural feature a few

points related to their structure merit further discussion. An alternative interpretation of the supposed attachment zones is the possibility that the apparently cytoplasmic filaments of the condensed area beneath the plasma membrane are in reality extracellular elastic unit fibers which at an angle of section oblique to the cell surface are projected on to the cytoplasmic area. This for instance might be the case in the left part of the area shown in Fig. 6. In the right part of the area however this can hardly be the case as the plasma membrane can be followed as a distinct line showing that the section has been cut almost perpendicular to its surface. In practically all instances studied a plasma membrane of an even increased density intervened between elastic and cytoplasmic filaments. This observation together with those previously mentioned seems to confirm that the dense areas represent more than accidental topographical relationships. *Kobayasi* (1961) studying the dermo-epidermal junction found delicate filaments probably of elastic tissue in the space between the corium membrane and the plasma membrane of the basal epidermal cells. This observation suggests a certain similarity between zones of epidermal and of endothelial attachments.

The finding of endothelial cell attachments to elastic tissue encourages the obvious thought about their significance in pathological changes of aging aortas in which a rarification of elastic tissue is known to occur. Our present knowledge permits only *conjecture at this point*.

Key to abbreviations used in the electron micrographs

E	Endothelial cell	pv	*Pinocytotic vesicles*
el	Elastic lamella	f	*Fenestra*
ei	Innermost elastic lamella of *tunica media*	Mn	Muscle cell nucleus
eb	Elastic branch	mf	*Myofilaments*
eu	Elastic unit fibers	ma	*Attachment of muscle cell to elastic tissue*
M	Smooth muscle cell	Mm	*Area of contact between muscle cells*
c	Collagen fibers	G	Golgi complex
cp	*Filamentous structure in elastic tissue*	a	Attachment of endothelial cell
fe	Filaments in endothelial cytoplasm	d	dense bodies in endothelial cells.

SUMMARY

Morphological features of the aortic tunica intima and media of young adult rabbits have *been investigated* as a basis for electron microscope studies on *epinephrine* induced arteriosclerosis. A subendothelial layer of appreciable width containing as its most conspicuous element numerous branching *elastic* fibers separates the endothelium from the tunica media. *The morphological evidence* [illegible]

[illegible] basal endothelial [illegible]

occur within the otherwise uniformly dense interior of elastic fibers especially in formalin fixed specimens Delicate filaments emanating from the surface of elastic fibers are interpreted as elastic structural units

REFERENCES

Berrian J H The electron microscopic structure of the rat aorta U S Naval School of Aviation Med Res Rept Proj No NM 001 057 10 03 1953

Buck R C The fine structure of endothelium of large arteries J Biophys Biochem Cytol 4 187–190 1958

Caesar R Edwards G A & Ruska H Architecture and nerve supply of mammalian smooth muscle tissue J Biophys Biochem Cytol 3 867 877 1957

Fawcett D W & Selby C C Observations on the fine structure of the turtle atrium J Biophys Biochem Cytol 4 63 72 1958

Karrer H E An electron microscope study of the aorta in young and aging mice J Ultrastruct Res 5 1–27 1961

Keech M K Electron microscope study of the normal rat aorta J Biophys Biochem Cytol 7 533 538 1960

Kobayasi T An electron microscope study on the dermo epidermal junction Acta Dermato Venereol 41 481 491 1961

Mark J S T An electron microscope study of uterine smooth muscle Anat Rec 125 473–493 1956

Moore D H & Ruska H The fine structure of capillaries and small arteries J Biophys Biochem Cytol 3 457–461 1957

Odland G F The fine structure of the interrelationship of cells in the human epidermis J Biophys Biochem Cytol 4 529 538 1958

Palade G E A study of fixation for electron microscopy J exp Med 95 285 298 1952

Palade G E Fine structure of blood capillaries J Appl Physics 24 1424 1953

Parker F An electron microscope study of coronary arteries Am J Anat 103 247–259 1958

Pease D C & Paule W J Electron microscopy of elastic arteries, the thoracic aorta of the rat J Ultrastruct Res 3 469 483 1960

Selby C C An electron microscope study of the epidermis of mammalian skin in thin sections J Biophys Biochem Cytol 1 429 443 1955

Takamatsu H Observations on the aortic media with the electron microscope Trans Soc Path Jap 47 1201 1207 1958

Thaemert J C Intercellular bridges as protoplasmic anastomoses between smooth muscle cells J Biophys Biochem Cytol 6 67 70 1959

The authors are pleased to acknowledge the excellent technical assistance of Mrs *F Skoldgaard Kristensen* Miss *A Pedersen* Mrs *B Thorsen* and Mrs *U Eberth* The study was aided by a grant from *The Danish State Research Foundation*

FROM THE GADE INSTITUTE DEPARTMENT OF PATHOLOGY UNIVERSITY OF BERGEN, NORWAY (HEAD PROFESSOR F WAALER M D)

RHEUMATOID PNEUMOCONIOSIS.

A case of Caplan's syndrome in a chalk-mine worker

By

JON LAMVIK

Received 31 viii 62

Caplan (1) in 1953, recorded a particular type of opacity in the chest radiographs of coal miners with rheumatoid polyarthritis. The shadows were numerous, small, some reaching a diameter of 1–2 cm, well defined and scattered over both lungs. About a quarter of the pneumoconiotic coal-miners with rheumatoid arthritis were found to have these opacities. Higher still was the frequency of massive pulmonary fibrosis, which is probably due to pneumoconiosis with secondary infection, most frequently tuberculosis.

Gough, Rivers & Seal (12) have described the pathological changes in the lungs from cases of Caplan's syndrome. Macroscopically small nodules were found with a concentric arrangement of darker and lighter areas. They resembled the infective type of nodules which are found in coal-miners with secondary infection in the lungs. Some of the nodules showed destruction in the lighter areas, while others were calcified. Microscopically the nodules were found to be made up of a central area composed of necrotic collagen containing dust that was often arranged in lines and circles. Around the necrotic layer a zone was found containing polymorphnuclear cells, lymphocytes, and macrophages. The peripheral part of the nodules showed collagen and fibroblasts, mainly concentrically arranged. In some nodules the fibroblasts showed pallisading, somewhat reminiscent of the pallisading found in subcutaneous rheumatoid nodules.

Similar radiological and/or pathological changes have been recorded in rheumatoid patients exposed to silica (5, 6, 7, 13), in a boiler scaler (3), in a foundry-worker (2), and in an asbestos-worker (16).

One case in a patient exposed to silica was presented in 1957 at a meeting of the Svenska Lakeresèlskapets sektion for Rheumatologi (11). We have not found any other report of this disorder in the Scandinavian medical literature. The syndrome seems to be of importance from a practical hygienic point of view as well as being of theoretical importance to the understanding of the pathogenesis of rheumatoid nodules.

The following case was recently examined at Gade's Institute

CASE REPORT

A 62 year old man had been working in a chalk mine and with quartz rock for 34 years until 1955 In 1954 he developed the first signs of rheumatoid arthritis stiffness and pain in the wrists and knees In 1955 he had typical symptoms and signs of rheumatoid arthritis He had to stop working because of the arthritis At this time he had no pulmonary symptoms and a chest x ray showed only slight thickening of the pleura

In 1956 he had swelling and impaired movement of the shoulders elbows and fingers exudate in the knees and stiffness of the ankles The muscles of the extr mities were atrophic A chest x ray showed bilateral infiltrations in the lungs He had no cough nor dyspnoea He was given Butazolidin orally and hydrocortisone injections into the shoulder and knee joints with some improvement

Within the following years he showed continuous signs of rheumatoid arthritis and was hospitalized several times and given physico therapy salicylic acid and gold injections

In 1959 he started to complain of dyspnoea

In February and September 1960 he had fever and pneumonic symptoms and was treated with antibiotics From this time on the dyspnoea became more marked

In October 1960 he was re admitted to the hospital because of arthritis symptoms At this time a chest x ray showed extensive fibrous infiltrates in both lungs with cavities in both upper lobes and in left lower lobe The tuberculin test was found to be negative Several specimens of sputum were cultured for TB with negative results A lymph node biopsy (a m Daniel) showed reactive hyperplasia

In the following 9 months he suffered continuously from dyspnoea and was given oxygen at times In the last months before he died he had no joint symptoms He died in August 1961 with combined cardio pulmonary failure

Laboratory tests

1956 Sedimentation rate 76 mm
White blood cell count 17000/mm^3
Serumprotein 7 25 gm per cent
Albumin/Globulin ratio 3 08/4 17

1959 Waaler Rose sheep cell agglutination test 1 320

Nov 1960 Antistreptolysin titre 1 60
Waaler Rose test 1 5120
Thymol turbidity 19 8 Mc Laghan units
Serumprotein 8 3 gm per cent
Albumin/Globulin ratio 4 4/3 9
Serum CO content 25 5 m eq/litre

Nov 1960 March 1961
25 sputums cultured for TB Negative

March 1961 Hgb 80 per cent WBC 10800/mm^3
Serum CO content 30 6 m eq/litre
Sputum Growth of pneumococci and staphylococci koagulase positive

July 1961 Serum CO_2 content 25 37 m eq litre
Sputum Growth of alcaligenes foecalis staphylococci koagulase positive and betahaemolytic streptococci

Autopsy Report

At the post mortem examination diffuse pulmonary fibrosis was found with multiple, small consolidations scattered over both lungs Both upper lobes showed marked fibrosis with destruction of the parenchyma Only fibrous trabeculi remained In the top of the left lower lobe several cavities, containing greyish, necrotic material, were noted The heart was enlarged, weighing 450 gm, with a hypertrophic right ventricle There were no signs of rheumatic valvular lesions The joints showed typical rheumatoid lesions



Fig 1 Microphotograph from the lung showing necrotic nodules surrounded by inflammation and fibrosis H + E × 90

Fig 2 The central necrotic area contains dust particles To the left of the necrosis the fibroblasts show pallisading reminiscent of the pallisading found in subcutaneous rheumatoid nodules H + E × 90

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however, showed the characteristic nodules with central necrosis surrounded by inflammation and fibrosis, as described by *Gough et al* (12) In some areas pallisading was noted reminiscent of subcutaneous rheumotoid nodules

Pulmonary granulomatous nodules have been described previously in patients with rheumatoid arthritis who have not been exposed to dust at work (10, 17), but as a rule the lungs either show no alterations, or interstitial fibrosis with chronic, reactive changes (9) In the reported cases of rheumatoid arthritis with non-pneumoconiotic changes in the lungs, rheumatoid granulomata have been found in other organs as well (16) It is unlikely that silica or other foreign particles have been of a pathogenetic importance in such cases In our case the lung tissue only showed necrotic changes It is possible that the silica present in the tissue has been of importance to the inflammatory, necrotic lesions The usual silicotic reaction, characterized by fibrosis and hyalinization and only moderate inflammatory reaction, appears to be modified in patients with rheumatoid disease with resulting necrosis of the pulmonary tissue and an inflammatory reaction, resembling rheumatoid lesions in the joints and skin

The pathogenesis of rheumatoid pneumoconiosis is obscure, as is that of silicosis The most recent theory concerning silicosis is that silica, instead of having a simple toxic effect on the tissues, combines with proteins and acts like an antigen *Cepellini & Pernis* (4) found that the acellular hyaline, silicotic tissue contained a fraction which was found to be identical to the plasma proteins, with a immuno-electrophoretic mobility similar to beta and gamma-globulins It is possible that these globulins are antibodies against silica or silica combined with human protein

It has been suggested that patients with rheumatoid arthritis are hypersensitive to tissue proteins (8) and the disease has been called an auto immune disease The rheumatoid factor has been considered as an antibody to gammaglobulin (14, 18) *Taylor & Shepherd* (18) and *Gluck & Waaler* (19) have found that factors in sera from rheumatoid patients are bound, in a specific way, to the rheumatoid nodules The rheumatoid factor is supposed to react with globulin complexes in the tissue (15)

Gough et al suggested that the inflammatory reaction in the lungs in cases of rheumatoid pneumoconiosis is caused by the necrosis of collagen due to silica It is possible also that patients with rheumatoid arthritis may show similar histological changes [illegible]

[illegible] reaction [illegible] be the cause of the necrosis as well as of the inflammation

Microscopical Examination

Sections from all parts of the lung showed small nodules surrounded by fibrosis (Fig 1) Normal lung structure was evident only in small areas The nodules had a characteristic structure with central necrosis where only hyaline and amorphous material, nuclear fragments and dust particles were seen The dust particles were found to be birefractive The necrotic areas were surrounded by a zone of inflammatory cells: lymphocytes, plasma cells, and some eosinophilic cells, but no giant cells Dust was also noted in this zone, both intra- and extracellular The zone was surrounded by a layer of collagenous connective tissue In some areas pallisading of the fibroblasts was evident (Fig 2) Most of the nodules were single, but some formed large confluent areas containing several nodules surrounded by inflammation and fibrosis Some arteries in the vicinity of the nedules showed marked endothelial proliferation with infiltration of mononuclear inflammatory cells In some areas in which the pulmonary structure had changed but little from the normal, pneumonia was evident In the fibrous septa haemosiderin granules were seen, probably the result of previous intrapulmonary haemorrhage

In the myocardium there was an increase in the amount of interstitial fibrous tissue, but there was no active inflammation No rheumatic or rheumatoid granulomata were found

DISCUSSION

The patient had been exposed to dust containing silica for a long period, 34 years He had had to stop working because of rheumatoid arthritis, which followed a typical course At this time a chest x-ray showed only minor changes It is, therefore, highly unlikely that impaired pulmonary function was the cause of the arthritis The signs and symptoms were clearly different from pulmonary osteo-arthropathy

In the next 4 years there was marked progress of the pulmonary fibrosis Destruction of lung tissue was found and a diagnosis of silicosis with secondary tuberculosis was considered

As stated by *Gough et al* (12) coal-miner s pneumoconiosis with secondary tuberculosis will cause gross and microscopical changes similar to the ones seen in rheumatoid pneumoconiosis Tuberculous infection will also modify the pathological lesions in silicosis and give destruction, cavities, and an inflammatory reaction

In the present case tuberculous infection seems to be very unlikely, as 30 sputums cultured for tubercle bacilli gave negative results and histological and bacteriological post mortem examinations gave no evidence of tuberculosis

The radiological changes in this case were not characteristic of silicosis and not typical of Caplan s syndrome The histological picture,

FROM STATENS SERUMINSTITUT COPENHAGEN, STREPTOCOCCAL DEPARTMENT CHIEF KNUD SKADHAUGE M D

INFECTION AND MALIGNANT TUMOURS

3 *Further Studies on the Effect on Tumours of Phage Lysates of Haemolytic Streptococci*

By

EBBE AHRENSBURG CHRISTENSEN

Received 15 ix 62

It has been demonstrated in previous studies (2 3) that a bacteria-free lysate of haemolytic streptococci, Group A, Type 12, produced by lysing the bacterial culture with streptococcal phages, inhibits growth and metastasization of Brown Pearce carcinoma A brief survey is given in those papers of the earlier reports concerning the antagonism between tumours and bacterial infections

The present paper deals with further studies on the effect on tumours of phage lysates of haemolytic streptococci

MATERIAL AND METHODS

Young male rabbits of a ...
sten the breeding f...
All the animals were ...
Transplantation to ...

Lysates of haemolytic streptococci Group A Type 12 No 3465 (local isolation number) were employed The bacteria were lysed by means of ...
in some preparations with sewage ...
sewage and strep phages (10) ...
tions was as described in a previou...
Dr Ebbe Kjems Statens Seruminsti...
in all cases (10)

The young animals were given the injections intraperitoneally 1 to 20 ml per injection Where treatment consisted of more than one injection this was administered as follows one injection weekly for three weeks two injections weekly for three weeks or three injections weekly for three w...

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This study has been aided by grants from the *P Carl Petersen Fund* and the Danish Cancer Society

SUMMARY

A case of rheumatoid pneumoconiosis in a chalk mine worker is reported The patient showed extensive fibrosis and cavitation in both lungs and clinically the case was suspected to be one of silicosis with secondary tuberculosis However, tubercle bacilli were not found and the histological changes in the lungs were similar to those reported in coal-miners with rheumatoid pneumoconiosis A possible pathogenetical interaction between silicotic tissue and rheumatoid factor is discussed

REFERENCES

1 *Caplan A* Certain unusual radiological appearances in the chest of coal miners suffering from rheumatoid arthritis Thorax *8* 29–37 1953
2 *Caplan A Cowen E D H & Gough J* Rheumatoid pneumoconiosis in a foundry worker Thorax *13* 181–184 1958
3 *Campbell J A* A case of Caplan s syndrome in a boilerscaler Thorax *13* 177–180 1958
4 *Cerpellini R & Pernis B* Presence of plasma globulins in the hyaline tissue in cases of silicosis Nature *181* 55 56 1958
5 *Colinet E* Un cas de panarthrite engainante Acta physiother rheum Belg 5 185–188 1950
6 *Colinet E* Polyarthrite chronique evolutive et silicose pulmonaire Acta physiother rheum Belg 8 37 41 1953
7 *Clerens J* Silicose pulmonaire et rhumatisme ou syndrome de Colinet Caplan Arch Belg Med Soc *11* 336 342 1953
8 *Dresner E* Some current concepts of the etiology of rheumatoid arthritis J Chronic Dis 5 612 629 1957
9 *Ellman P & Ball R E* Rheumatoid Disease with joint and pulmonary manifestations Brit Med J 2 816 820 1948
10 *Ellman P Cudkowicz L & Elwood J S* Widespread serous membrane involvement by rheumatoid nodules J Clin Path 7 239 244 1954
11 *Glomme J* Caplan s syndrom Svenska reumatologisk forening Nord Med 57 231 1957
12 *Gough J Rivers D & Seal R M E* Pathological studies of modified pneumoconiosis in coal miners with rheumatoid arthritis (Caplan s syndrome) Thorax *10* 9 18 1955
13 *Martin F & Lallet G H* Pneumopathies chroniques et rhumatisme Schweiz med Wchnschr *83* 776 782 1953
14 *Mellors R C Helmer R Corcos J & Korngold L* Cellular origin of rheumatoid factor J Exper Med *110* 875 886 1957
15 *Mellors R C Nowoslawski A & Korngold L* Rheumatoid arthritis and the cellular origin of rheumatoid factors Am J Path 39 533 546 1961
16 *Richards A G & Barrett G M* Rheumatoid lung changes associated with asbestosis Thorax *13* 185–193 1958
17 *Sl ogrand A* Visceral lesions in rheumatoid arthritis Acta rheum scandinav 2 17 30 1956
18 *Taylor H E & Sheperd W F* The immunohistochemical interaction of autologous rheumatoid serum with subcutaneous rheumatoid nodules Lab Invest 9 603 612 1960
19 *Waaler E* The immuno pathology of the collagen diseases Acta path et microbiol scandinav Suppl *154* 29 39 1962

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MATERIAL AND METHODS

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The young animals were given the injections intraperitoneally, 1 to 20 ml per injection Where treatment consisted of more th

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same examiner On the basis of autopsy findings the animals were divided into three groups according to the occurrence of metastases (A) and three groups according to the quantity of tumour tissue (B) (3)

A 1 Tumour growth in the peritoneum only
 2 Tumour growth in the peritoneum and invasion into the diaphragm to the pleura
 3 Metastases at one or more sites

B 1 No or little tumour tissue
 2 Moderate quantity of tumour tissue
 3 Large quantity of tumour tissue

In the experiments with mice 300 albino mice of Statens Seruminstitut Breed C were used (this breed has been inbred for more than twenty years) All of the mice weighed from 28 to 30 grammes at the time of transplantation and distribution into the experimental groups was made at random

Subsequent passages of Ehrlich ascites carcinoma were used Each animal was given 0 2 ml undiluted ascites fluid from a mixture of fluid from ten donors (The tumour was provided by Dr *Helge Lund* The University Institute of Hygiene Copenhagen who has had the tumour in mice transfers for more than ten years

The mice were given intraperitoneal injections of bacterial lysate or placebo (serum broth) every alternate day from the day after transplantation

The survival time after transplantation was used as indication of the effect of treatment Three mice which did not die spontaneously were sacrificed 60 days after transplantation Autopsy was performed on all the mice

The mice were treated with the same bacterial lysates and placebo preparations as the rabbits, however physiological saline was given as placebo to 20 mice instead of serum broth

TABLE 1

	No of rabbits at commencement of experiment	No of rabbits dead before 12th day after transplantation	No of rabbits with adequate observation period	No of rabbits without vital tumour tissue at autopsy
Treated with optimal dose				
Lysate	52	0	52	20
Placebo	33	1	32	0
Treated with two to four times the optimal dose				
Lysate	54	29	25	9
Placebo	33	5	28	0
Treated with half to quarter of optimal dose				
Lysate	30	0	30	1
Placebo	27	1	26	0
Treated with one sixth to one tenth of optimal dose				
Lysate	7	0	7	0
Placebo	6	0	6	0
Untreated control animals	56	1	55	0

RESULTS

Rabbit experiments

In the *first series of experiments* the individual dose varied from 1 to 20 ml The first injection was given on the 2nd day after transplan-

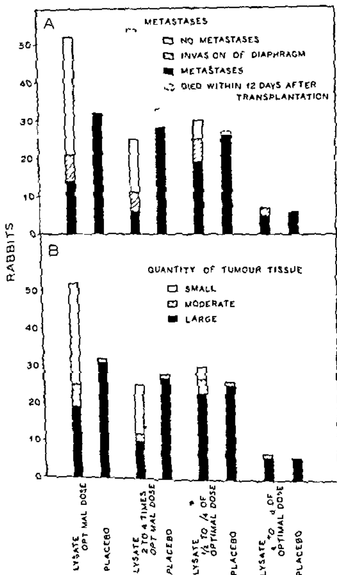


Fig 1
Metastasization and quantity of tumour tissue using doses of varying size

tation and the treatment continued with three injections per week for 3 weeks. All the surviving animals were sacrificed on the 25th or 30th day after transplantation

The best tumour inhibiting effect was obtained with the largest dose which did not cause deterioration of the general condition of the animals as compared with that of the untreated tumour bearing

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groups However, the few animals that survived this intense treatment showed the same frequency of metastases and tumour development as those given the optimal treatment (Figs 1 A and 1 B)

Much more inferior results were obtained with a half or a quarter of the optimal dose, and when the dose was reduced to a sixth or a tenth of the optimal dose, the results were the same as following placebo treatment However, the number of animals in this group was very small (Figs 1 A and 1 B)

In the first set of experiments autopsy revealed that there was no vital tumour tissue in 30 out of 261 animals (macroscopic examination) All 30 had been treated with bacterial lysate, 20 with the optimal dose, 9 with two to four times the optimal dose and 1 with half the optimal dose (Table 1)

In the *second series of experiments* the number of injections per rabbit varied The first injection was given two days after transplantation The dose administered was as large as possible without causing deterioration of the general condition of the animals during the treatment

Four groups of young rabbits were given one, three, six or nine to ten injections of bacterial lysate, respectively, one group was injected with human serum broth and one was not injected

In 13 animals given one injection of bacterial lysate, the rate of growth and metastasization was exactly the same as found in 18 placebo treated and 24 untreated rabbits (Figs 2 A and 2 B)

In the three groups in which several injections of bacterial lysate were given, inhibition of tumour growth in the peritoneum and also blockage of the ability to metastasize were seen in a considerable number of instances This blockage of the metastasizing ability was also observed in some of the animals which, in spite of the treatment had moderate or large quantities of tumour tissue in the peritoneum (Figs 2 A and 2 B)

This series of experiments showed that a single injection of bacterial lysate given a few days after transplantation, had no effect even if it was as large as possible without deteriorating the general condition of the animal Continued treatment, however, resulted in inhibition of both growth of tumour and of metastasization The material is too small to determine whether there was any actual difference in the results of treatment with three, six or nine to ten injections

In 10 out of 94 animals no vital tumour tissue was found at autopsy Nine of these had been treated with bacterial lysate, 2 with three injections 4 with six injections and 3 with nine or ten injections One of the control animals which had received three injections of human serum broth presented no trace of tumour growth at autopsy (Table 2)

In the *third series of experiments* treatment was instituted at various times after transplantation

rabbits This optimal dose varied from 2 to 10 ml according to the preparation used (Table 1 and Fig 1 A)

The largest doses, *viz* 15 and 20 ml, caused deterioration of the general condition in all cases, and the mortality rate following treatment was very high (Table 1 and Fig 1 A)

Out of 54 animals given a dose two to four times greater than the optimal dose, 29 died as the result of the treatment before the 12th day after transplantation, *i e* before the tumour had developed sufficiently to allow a comparison with the placebo-treated or untreated

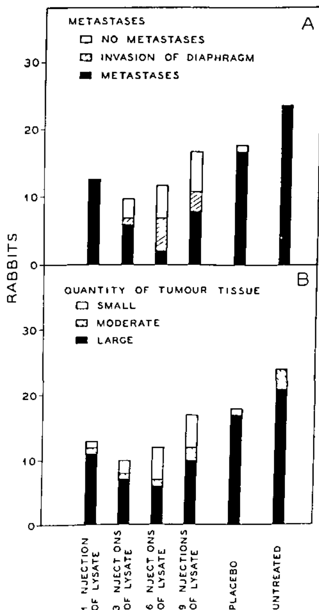


Fig 2

Metastasization and quantity of tumour tissue using varying numbers of injections

In 12 out of 89 animals in this series, no vital tumour tissue could be found at autopsy As regards 10 of these animals, treatment with bacterial lysate had been started between the 2nd and 8th day, while in one animal lysate treatment was commenced on the 15th day Placebo treatment was started in one animal on the 15th day, and autopsy of this animal revealed no traces of tumour development (Table 3)

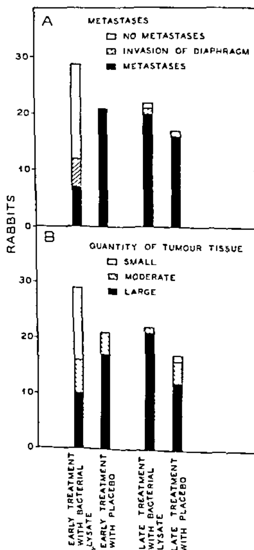


Fig 3
Metastasization and quantity of tumour tissue using varying times for commencement of treatment

TABLE 2

	No of rabbits at commencement of experiment	No of rabbits dead before 12th day after transplantation	No of rabbits with adequate observation period	No of rabbits without vital tumour tissue at autopsy
Treated with one injection of bacterial lysate	14	1	13	0
Treated with three injections of bacterial lysate	10	0	10	2
Treated with six injections of bacterial lysate	14	2	12	4
Treated with nine or ten injections of bacterial lysate	18	1	17	3
Placebo	20	2	18	1
Untreated rabbits	24	0	24	0

Using the same technique, it was found previously (*Christensen & Kjems* 1959) that there was pronounced tumour growth in the peritoneum between the 4th and 7th day and macroscopically visible metastases between the 9th and 13th day after transplantation in almost all of the untreated or placebo-treated animals

In this series, 21 untreated animals were sacrificed between the 4th and 15th day after transplantation The rate of growth of tumour tissue and the time of occurrence of visible metastases were found to be in accordance with previous observations 13 animals killed between the 4th and 11th day all had significant growth of tumour tissue in the peritoneum In one of the animals the tumour tissue had invaded the diaphragm but none of the animals had any macroscopically visible metastasization Three animals killed on the 11th day and 5 on the 15th day after transplantation all had metastases and moderate or large quantities of tumour tissue in the peritoneum

The rest of the animals in this series, *viz* 89 young rabbits, were divided into seven groups Treatment was commenced on the 2nd, 5th, 7th, 8th, 10th, 11th and 15th day after transplantation respectively, and continued with three injections per week until ten injections had been given or the rabbit had died

In the four groups in which treatment with bacterial lysate was commenced on the 2nd, 5th, 7th and 8th day (early treatment), inhibition of tumour growth and metastasization were seen in a number of the animals There was no inhibition of tumour growth in the three groups in which treatment was instituted on the 10th, 11th and 15th day (late treatment) (Figs 3 A and 3 B)

No difference could be demonstrated between the results of treatment within the four first-mentioned groups and within the three latter groups (Figs 4 A and 4 B)

In 12 out of 89 animals in this series, no vital tumour tissue could be found at autopsy. As regards 10 of these animals, treatment with bacterial lysate had been started between the 2nd and 8th day, while in one animal lysate treatment was commenced on the 15th day. Placebo treatment was started in one animal on the 15th day, and autopsy of this animal revealed no traces of tumour development (Table 3)

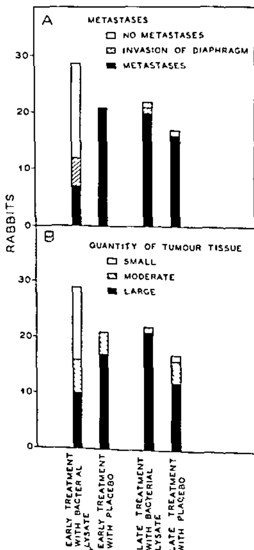


Fig 3
Metastasization and quantity of tumour tissue using varying times for commencement of treatment

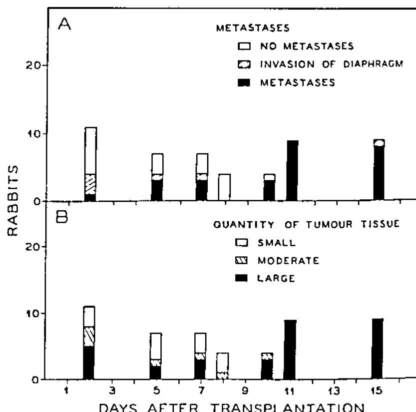


Fig 4

Metastasization and quantity of tumour tissue in the individual groups of animals using varying times for commencement of treatment

TABLE 3

	No of rabbits at commencement of experiment	No of rabbits dead before 12th day after transplantation	No of rabbits with adequate observation period	No of rabbits without vital tumour tissue at autopsy
Treatment commenced between 2nd and 8th day after transplantation				
Lysate	30	1	29	10
Placebo	23	2	21	0
Treatment commenced between 10th and 15th day after transplantation				
Lysate	23	1	22	1
Placebo	17	0	17	1

In order to exclude the possibility that the negative result of treatment with a single injection of bacterial lysate was due to the injection having been given too early in the course of the development of the tumour, 6 young rabbits were given one injection on the 8th day after transplantation At autopsy all 6 animals were found to have metastases and moderate or large quantities of tumour tissue

In the second and third series of experiments combined 10 out of 199 animals died before the 12th day after transplantation These were excluded from the material, since they could not be compared with the others from the point of view of quantity of tumour tissue and metastasization Treatment with bacterial lysate had been commenced in 6 of these animals and 4 were under treatment with placebo (Tables 2 and 3)

During the production of lysates from haemolytic streptococci, contamination occurred a few times with Gram negative bacteria, twice with Escherichia coli When such contamination was pronounced the preparation completely lost its inhibiting effect on the Brown-Pearce carcinoma and at the same time became considerably more toxic These changes were not due to infection of the experimental animals, since they were found even when the preparation had been rendered sterile by filtration before use

Some cases of diffuse peritonitis with Gram negative rods occurred among the rabbits as all the injections were given by the intraperitoneal route In some of these animals there was inhibition of the local tumour because of the peritonitis, but the organic metastases were not affected by such infection

Mouse experiments

In the experiments on mice with Ehrlich ascites carcinoma the bacterial lysates had no effect on the median survival time after trans-

TABLE 4

	No of mice	Median survival time
Untreated	87*	12 7 days
Dose per gramme as optimal dose in young rabbits		
Lysate	40	12 0 days
Placebo (serum broth)	30	11 8 days
(physiological saline)	10	12 9 days
Dose per gramme twice the optimal dose in young rabbits		
Lysate	58†	11 8 days
Placebo (serum broth)	40	9.3 days
(physiological saline)	10	14 4 days
Dose per gramme three times the optimal dose in young rabbits		
Lysate	10	10 1 days
Placebo (serum broth)	10	10 2 days

[illegible] of tumour [illegible] from tumour growth 33 days after transplantation

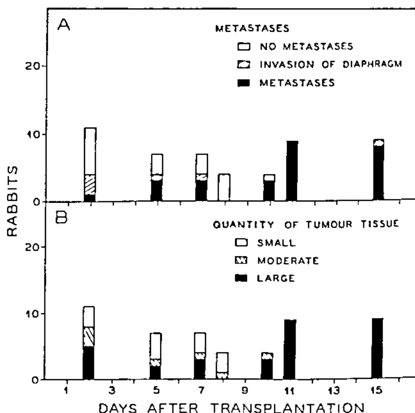


Fig 4

Metastasization and quantity of tumour tissue in the individual groups of animals using varying times for commencement of treatment

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Placebo (serum broth)	40	9 3 days
(physiological saline)	10	14 4 days
Dose per gramme three times the optimal dose in young rabbits		
Lysate	40	[illegible]

[illegible] tumour growth 33 days after transplanta[illegible]

plantation, even though the smallest of the doses per gramme body weight was just as large as the optimal dose given to the young rabbits, and the largest dose was three times bigger (Table 4)

DISCUSSION

In agreement with previous studies (*Christensen & Kjems* 1959), the present experiments have shown that bacteria-free lysates of haemolytic streptococci can inhibit both development of metastases and local growth of Brown-Pearce carcinoma

With the technique employed here, repeated injections of bacterial lysate must be given in order to demonstrate this effect on the tumour, and treatment must be instituted before the 10th day after transplantation

The unpurified preparations are toxic and cause general deterioration in doses which are only slightly higher than those which inhibit the tumours There is considerable variation in the toxicity and in the ability to inhibit tumour growth from one preparation to another However, on the basis of the material available, it cannot be determined whether the tumour-inhibiting effect and the toxicity are proportional

As mentioned, a single injection had no effect, even when the dose was as large as possible without affecting the general condition of the experimental animals This is of interest, since it shows that a non-effective injection does not interfere with the effect of subsequent injections This is in contrast with the results obtained with Shear's polysaccharide and allied substances from Gram-negative bacteria on adult mice with tumours With these substances an effect is seen only after the first injection, because the mice and the tumour tissue rapidly acquire increased resistance to the substances (16) Preparations of the "Coley Mixed Toxins" type also cause increased resistance after the first injection (7)

It has been demonstrated with Shear's polysaccharide that the mouse tumours must be of a certain age and size to give reaction in the form of haemorrhagic necrosis after injection of the substance This depends on the fact that the action of the polysaccharide is due mainly to its ability to induce a marked hypotension in the experimental animals, and consequently the effect will not be significant until the tumour is vascularized (5, 4, 1) In contrast, the effect of the lysates seems to be without relation to the vascularization A single injection of bacterial lysate had no effect whether received on the 2nd or on the 8th day after transplantation, and a series of injections had the same tumour-inhibiting effect whether it was started on the 2nd or on the 8th day On the 2nd day a small amount of tumour tissue was found in the control animals and the tissue had no vascularization – on the 8th day the rabbits had a considerable amount of tumour tissue in the peritoneum and the tissue was vascularized

On account of the general condition of the animal, the single injection given in the present experiments could not be more than about one-third of the dose found to be optimal if the treatment was distributed in the form of six to ten injections This seems sufficient to explain the fact that a single injection was without effect

Another eventuality is that the tumour inhibiting effect might be connected with an immunizing process, and that repeated injections are required for either the animal or the tumour to form antibodies against the bacterial lysate before inhibition of the tumour commenced The present experiments carried out with Brown-Pearce carcinoma neither exclude nor support this possibility

In this connection it should be mentioned that development of antibodies against Brown Pearce carcinoma is of significance as regards the course of tumour growth in adult rabbits (8, 9, 13, 14, 15)

In the present study, young animals were employed because these animals have a poorer ability to form antibodies than the adults In the experiments a fairly uniform tumour development occurred in young animals which were less than 18 days and more than 7 days old at time of transplantation Immunological tolerance can be provoked in young rabbits up to 17 days after birth by means of bovine albumin, ovalbumin and human gamma globulin (18) The uniform and rapid growth of Brown-Pearce carcinoma and the almost complete absence of tendency to regression in the young, in contrast to the great tendency in the adults, may possibly be due to immunological tolerance to the tumour having been induced by transplantation and to this tolerance being maintained by the growing tumour

It seems to be much more difficult to provoke immunological tolerance to antigens of bacterial origin (18, 17) Nothing is known about the ability of lysates of haemolytic streptococci to induce immunological tolerance For this reason it is impossible to decide whether an antigen-antibody reaction caused by the lysates in the young rabbits play any role in the tumour-inhibiting effect

If treatment was commenced later than the 8th to 10th day after transplantation, the only demonstrable effect was a higher mortality Almost all of the untreated young rabbits with Brown-Pearce carcinoma had a good general condition and showed normal increase in weight 9 to 11 days after transplantation However, autopsy at that stage revealed that there were considerable quantities of tumour tissue in the peritoneum, frequently invasion of the diaphragm, and often visible metastases in the organs Treatment of animals having such large quantities of tumour tissue in relation to the [illegible]

[illegible] an *in vitro*/*in vivo* technique, were able to prevent the take of Ehrlich ascites carcinoma by means of haemolytic streptococci and an extract of haemolytic streptococci (11, 12) The tu-

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8 *Kidd J G* Distinctive substance associated with Brown Pearce rabbit carcinoma I J Exper Med *71* 335 1940
9 *Kidd J G* Distinctive substance associated with Brown Pearce rabbit carcinoma II J Exper Med *71* 351 1940
10 *Kjems E* Studies on streptococcal bacteriophages II Acta path et microbiol scand nav *42* 56 1958
11 *Koshimura S Murasawa K Nakagawa E Ueda M Bando Y & Hirata R* Experimental anticancer studies III Japan J Exper Med 25 93 1955
12 *Koshimura S & Shoin S* Experimental anticancer studies Part 13 Gann *51* 309 1960
13
14
15 stinctive
16 Cancer Inst 4 461 1944
17 *Smith R T* Studies on the mechanism of immune tolerance In "Mechanisms of Antibody Formation Proc of a symp Prague May 27 31 1959 p 313 328
18 *Smith R T & Bridges R A* Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens J Exper Med *108* 227 1958
19 *Zahl P A* Action of bacterial toxins on tumors VIII J Nat Cancer Inst *11* 279 1950

mour cells were exposed to a very strong effect *in vitro*, after which transplantation to mice was used to demonstrate whether sufficient vital tumour cells were left to cause growth of the tumour in the mice However, the method provides no information regarding the dose able to inhibit the tumour cells *in vivo*

In the present study the negative outcome of the attempt to influence Ehrlich ascites carcinoma with a lysate of haemolytic streptococci shows that the ascites carcinoma *in vivo* is less sensitive to streptococcal lysates than the Brown-Pearce carcinoma It cannot be determined with the preparations used whether the ascites carcinoma *in vivo* can be inhibited at all by streptococci, since the survival time of both the lysate-treated and the placebo-treated mice was decreased considerably by a further increase of the dose

SUMMARY

Brown-Pearce carcinoma can be inhibited in young rabbits by lysates of haemolytic streptococci The inhibiting effect can be demonstrated provided that

1 The doses administered are so large that they approach the toxic level
2 The treatment comprises a series of injections distributed over three weeks
3 The treatment is instituted between the 2nd and 8th day after transplantation

Lysates of haemolytic streptococci do not prolong the median survival time of mice with Ehrlich ascites carcinoma

The experimental results are discussed in relation to the effect of toxins from Gram-negative bacteria on tumours in mice and to the effect of streptococci on Ehrlich ascites carcinoma demonstrated by Koshimura et al

REFERENCES

1 *Algire G H Legallis F Y & Anderson B F* Vascular reactions of normal and malignant tissues in vivo V J Nat Cancer Inst *12* 1279 1952
2 *Christensen E A* Infection and malignant tumours I Acta path et microbiol scandinav , *46* 285, 1959
3 *Christensen E A & Kjems E* Infection and malignant tumours II Acta path et microbiol scandinav *46* 296 1959
4 *Diller J C* Degenerative changes induced in tumor cells by Serratia marcescens polysaccharide Cancer Res *7* 605 1947
5 *Duran Reynals F* Reaction of transplantable and spontaneous tumors to blood carried bacterial toxins in animals unsuspectible to the Shwartzman phenomenon Proc Soc Exper Biol & Med *31* 341 1933
6 *Havas H F Donnelly A J & Levine S I* Mixed bacterial toxins in the treatment of tumors III Cancer Res *20* 393 1960
7 *Havas H F Groesbeck M E & Donnelly A J* Mixed bacterial toxins in the treatment of tumors I Cancer Res *18* 141 1958

(1927) The temperature changed the form of the colonies, the production of pigment, the quality and quantity of bacterial growth in fluid media the formation of volutin and flagella, the fermentative reactions, the coagulase production, the virulence, etc

Billing (1956) observed a change in bacterial morphology when the incubation temperature was lowered from 37° C to 25° C

Olsen (1949) isolated large fusiform microorganisms and classified them tentatively on account of the large forms, into Spironema Sanarelli group Culturally, morphologically, and biochemically, these organisms seem to be identical with the bacteria described in the present paper

MATERIALS AND METHODS

More than forty strains of pleomorphic Bacterium anitratum were isolated recently from a clinical material especially from urine and from swabs of gynaecological ... became predomi... emperature and ... d in the micro... be due to the ... in the patients had been exposed before the samples for bacteriological examinations had been taken Nevertheless a closer investigation revealed that the cell forms evidently were dependent on the incubation temperature The fusiform shapes in the cultures grown at 37° C were supposed to be due to the dissociation of the divison process from the process of growth of these microorganisms It seemed that the division of the strains examined was inhibited at 37° C, while growth still continued so that oversized and deformed cells developed

Among these forty strains which all changed their form with the incubation temperature two strains each with a different reaction to the temperature of 37° C, and with a different reaction to ... study on ... factors ... shaped fo ... experimen... out to see whether the same morphological changes could be obtained also in experimental animals

The first strain H was isolated in a pure culture from the urine of a middle aged patient with the clinical diagnosis Carcinoma colli uteri Strain P was isolated from a mixed culture as a contaminant

After the preliminary investigations both strains were kept lyophilized for about two months before they were subcultured every two weeks us... agar plates ...

In order to test the ... both strains and to det... logical changes three ... First both strains w... perature and in the te...

... 34° and 41° C for the same periods of time Thus the effect of all temperatures in the above mentioned interval was investigated in the cultures which

THE INFLUENCE OF TEMPERATURE ON THE SIZE AND SHAPE OF BACTERIUM ANITRATUM

By

BRONKA BRZIN[1]

Received 10 viii 62

The present paper is a study on the influence of conditions of cultivation on bacterial cytology The effects of physical and chemical agents on the cell growth of microorganisms often reflect processsess preparing the organisms for division or responsible for division itself The studies, especially on the factors which inhibit cell division, could therefore be useful in order to obtain information about the pathways of these processes

The temperature is an agent influencing cell division, and this effect has been used successfully to synchronize the cell division in populations of protozoa (*Scherbaum & Zeuthen* 1954, 1955) and bacteria (*Lark & Maaloe* 1954) The experiments discussed below were carried out with the intention to determine whether the temperature, at which the microorganisms were grown, might be responsible for the pronounced changes in size and shape observed in Bacterium anitratum Pleomorphism in this species is a known fact, but no consistent relation between the morphological behaviour and the conditions of cultivation has been demonstrated (*Seeliger* 1952)

Many bacteria change their form in an unfavourable environment, and in the early bacteriological evidence many interesting observations of such changes are discussed (*Lohnis* 1922) In most cases the changes described were induced by chemical or unknown agents Later, similar changes were observed under poor growth conditions (*Ørskov* 1947)

It is noteworthy that very early a low temperature was found to be a factor inducing morphological changes in bacteria (*Almquist* 1909)

The stimulative effect of a high temperature on bacterial variation was studied by many early authors and reviewed already by *Hadley*

This work has been made possible through the hospitality of Statens Serum institut and I wish to express my sincere thanks to its director *Preben von Magnus* M D and to its members especially to *P Holm H Lautrop* and *F A Freundt* My thanks are due also to Prof *E Zeuthen* Biologiske Institut Carlsbergfoundation who stimulated this work with his suggestions and allowed me to use the temperature gradient water bath in his laboratory

I should like to thank also *R Ceroni* who made the Feulgen staining

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mouse and one guinea pig, respectively, were sacrificed every 24 hours Smears from the peritoneum blood, and the internal organs of the sacrificed animals were examined microscopically, and cultivated on 10 per cent blood agar plates

RESULTS

Differences in the cell morphology were significant and consistent in cultures incubated at temperatures below 34°–35° C, as compared with those incubated above this temperature For the sake of clarity only the results obtained at room temperature and at 37° C are described in this paper

1 *Growth at Room Temperature*

A *Macroscopical picture.* After 24 hours of aerobic growth the colonies of both strains were circular, grey, low convex, smooth, shining, displaying an unbroken outline The diameter of each colony varied from one to 2 mm but it enlarged slightly during the next 24 hours of incubation Upon prolonged incubation at room temperature (second week and later), tongue-like protrusions, similar to those of Myxobacteria, spread from the border of some of the colonies, especially in strain H

B *Microscopical picture:* Wet mounts and stained preparations taken from the centre or from the border of the colonies which had been subcultured at room temperature from the room temperature cultures, showed (Fig 1, 2) cocci, ovoids (about 0 5 × 1 μ in diameter) and rare, short bacilli (about 1 × 3 μ in diameter) Longer bacilli were found only rarely, especially in strain H Slides prepared from various waves and also from the extreme border of the tongue-like protrusions, showed the same forms and dimensions Some bigger and rounder cells were the only exceptions found at the end of the first week during prolonged observations (for 3 months) of the cultures incubated at room temperature Some of these cells measured up to 4 μ in diameter and displayed a darker central or paracentral area They might correspond to microcysts in other species After subcultivation they always reverted to small coccoid and bacillary forms within 24 hours of incubation at room [illegible]
the cultures inc[illegible]
filaments, the s[illegible] at room temperature showed the same coccoid and short bacillary forms as the subcultures taken [illegible]
cultures [illegible]

[illegible] 2 to 10 rect- [illegible] rounded up later so that long chains of oval bacilli were found in the following hours

were subcultured from colonies cultured at the same and at different incubation temperatures

In the second group of experiments the cultures were not subcultured on a fresh medium but only the temperature of incubation was changed Microscopical examinations were made at various intervals after the transfer

The third group of experiments was carried out as follows From the border of the colonies the bacteria were smeared on a (free) part of the surrounding medium Some of these plates were immediately transferred to other temperatures the others on various later occasions At various time intervals the new growth was compared with the old one, macroscopically and microscopically

Besides these series of experiments both strains were incubated at 37°, at 25° C and at room temperature continuously for a longer period (up to one month at 37° and at 25° C till 3 months at room temperature) These cultures were examined every two hours during the first day, every day during the first week then once a week

In order to test the possible role of other factors inducing big fusiform cells in these organisms 24 hour cultures of both strain previously grown at 37° C and at room temperatures were subcultured to various media and incubated at these two temperatures After 24 48 and 72 hours the form of the colonies and the microscopical morphology of both strains were compared with the macroscopical and microscopical morphology of corresponding cultures grown on nutrient agar for the same length of time For this investigation the following groups of media were used

1 To test the nutrition factors various enriched (10 per cent blood agar, placenta agar, glucose agar, etc) and some chemically deficient (Sohngen medium without a source of carbon Corn meal agar) media were used
2 To test the influence of various pH of the media nutrient agar adjusted to pH 5 5 and 8 5, and Kulp medium with a pH of 6 1 were used
3 To test the influence of chemical factors which inhibit growth of the microorganisms examined bile agar, telurit agar, and nutrient agar with 0 5 per cent oleic acid were used
4 To test the action of antibiotics experiments with penicillin terramycin and chloromycetin were instituted The paper disc method (Whatman No 2 filter paper diameter of 6 5 mm) was used The concentrations were as described by *Schaub & Foley* (1952) For comparison penicillin was also incorporated into the medium (blood agar) in a concentration of 0 5 unit/ml
5 To test the physical factors of growth physically deficient media were used For this purpose various media were dried for 12 24 and 48 hours at 37° C and at room temperature thus making them too dry for optimal growth or in the extreme cases too dry for any macroscopically visible growth of these microorganisms
6 To test the anaerobic growth conditions
A The plates were incubated in anaerobic jars in the presence or absence of CO_2 After 5 days of incubation the smears were made and the plates were incubated for the next 5 days under the same conditions Two or three hours of growth at room temperature under aerobic conditions elapsed between the anaerobic incubation periods of 5 days
B Inoculations were made under the surface of solid and semisolid media

Besides being inoculated on and into these solid and semisolid media both strains were inoculated into some fluid media (infusion broth infusion broth enriched with a few drops of serum ascites or blood Brewer thioglycollate medium) incubated at the temperatures mentioned above and examined at the same time intervals macroscopically and microscopically

The survival time of both strains at 37° C and at room temperature was determined by prolonged incubation (up to one month at 37° C up to 6 months at room temperature) on thick 10 per cent blood agar plates Every day of the first week subsequently every third day subcultures from each of the two temperatures were made The subcultures were observed for 72 hours before being discarded as negative

Animal experiments were carried out as follows A 48 hour colony of each morphological form of the two strains was suspended in 0 5 ml af infusion broth and immediately injected intraperitoneally into three guinea pigs and three mice One

B *Microscopic picture* As regards the inoculum taken during the logarithmic phase of growth at room temperature, the changes in length were seen already during the first hours of growth, but the cells became only slightly wider (Fig 3, 4) Growth in length continued during the next hours of incubation After the first 12 hours of incubation the polymorphism began to be apparent also in width The long filaments of strain H swelled in the middle but remained threadlike and tapered towards both ends (Fig 5) In strain P the form of the swellings was more irregular (Fig 6) The fusiform central and paracentral swellings (characteristic of strain H) and terminal and paraterminal swellings (characteristic of strain P) continued to grow within the following 24 hours of incubation at 37° C By the end of this time the length of many filaments measured up to 50 μ or above After prolonged incubation at 37° C (for three days), many poorly stained large filaments with irregular outlines were found

As regards the inoculum taken from cultures growing at 37° C, no essential changes were seen in 24 or 48 hours subcultures growing at the same temperatures

Corresponding changes were observed if the cultures were not transferred to a fresh medium but only the temperature of incubation was changed If the cells were still young when the incubation temperature was raised or lowered, they changed in 24–48 hours In cultures more than 24 hours old the changes occurred more slowly and were never complete Many ghost like cells remained after the transfer to room temperature in cultures which had been grown at 37° C for a longer time The interval in which the changes might still occur shortened if the media were dried up

Observations were similar in the third group of experiments

3 *Temperature-Resistent Colonies*

Under the conditions described above the changes were reversible and in accordance with the incubation temperature during two years of experimental work The only exceptions were some secondary colonies which proved to be temperature resistent They developed during the second week of incubation on and between the ordinary colonies grown at 37° C In these colonies cocci and short bacilli prevailed in spite of their growth at 37° C These secondary colonies retained their morphological features also during several subcultivations at 37° C Only gradually typical long cells began to appear in the subcultures of these colonies Within three weeks some of them completely reverted to the typical colonies which changed their microscopical form with the incubation temperature In other colonies the reversion took a longer time (from 2 till 3 months) No permanent temperature resistent colonies were found

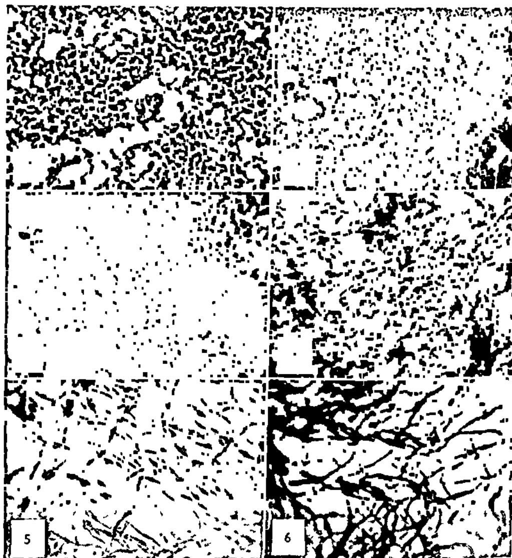


Fig 1 6

Gram stain Magnification × ca 1000

Fig 1 Strain H growing at room temperature for 48 hours
Fig 2 Strain P growing at room temperature for 48 hours
Fig 3 Strain H growing at 37° C for 6 hours
Fig 4 Strain P growing at 37° C for 6 hours
Fig 5 Strain H growing at 37° C for 48 hours The swellings are fusiform They are formed in the middle of the filaments
Fig 6 Strain P growing at 37° C for 48 hours Note clubed cells (the enlargements are formed at the end of the filaments)

2. *Effect of Higher Incubation Temperature* (37° C)

A *Macroscopic picture* The 24-hour-colonies of strain H were larger, of strain P smaller, than the corresponding colonies of each strain growing at room temperature In other respects they corresponded to those incubated at room temperature

8 The survival time for strain H at 37° C was about 3 weeks, for strain P only 4 days At room temperature, strain H survived for 4–6 months, strain P for 6–8 weeks After prolonged incubation at room temperature (about 8 weeks), strain P could no longer be subcultured directly to 37° C However, this was successfully carried out after an intermediate subcultivation at room temperature

9 Animal experiments Microorganisms were found in smears and in cultures from the sacrified animals In smears, no changes in the form of the injected microorganisms were found except in animals which had received small coccoid forms from the cultures grown at room temperature Many medium sized, fusiform bacilli, less than half as long as those grown on solid media at 37° C for the same period, were found in the smears taken from the peritoneum and from the internal organs of the animals, especially from the ones, sacrificed 48 hours after the intraperitoneal injection

Microorganisms cultivated after the passage through the experimental animals grew in their ordinary forms according to the temperature of incubation to which they were exposed

DISCUSSION

The factors giving rise to the development of the abnormal forms are worth investigating since they may also throw light on the still much discussed problem of the regeneration cycle Various bizarre forms were considered to be due either to degeneration or to involution of bacteria, or to represent stages in the regeneration cycle (*Eisenstark et al* 1950) Subsequent investigation showed that in some cases these phenomena were effected by some adverse factor (*Tulasne* 1949) In our experiments changes in size and shape of Bacterium anitratum examined were found to be related to the incubation temperature Gross changes in size resulted from the dissociation of division from growth by the incubation temperature of 37° C, division being inhibited while growth still continued The dependence on temperature of cell growth of these microorganisms is evidently different from that of cell reproduction

The ability of growth to continue varies at different incubation temperatures Suitable shifts, either to a higher or to a lower temperature,

rowth This inhibition of growth is

In the extreme cases, *i e*, *if division*

no change in size is observed No fusiform shapes are found when the cells are cultivated in anaerobic conditions or on a physically or chemically inappropriate medium inhibiting growth even if they are exposed to the deleterious action of 37° C This finding is in accordance with the results obtained by *Eisenstark et al* (1950) who on the supposition that toxic agents operate to distort the cell morphology, added many inhibitory substances to basal

4 *Microorganisms which Were more Sensitive to the Influence of Higher Incubation Temperatures than the Ordinary Ones*

In contrast to the temperature-resistent colonies described above, some were found which were more sensitive to temperature than the ordinary ones These colonies appeared, as a rule, during the second day of incubation at 37° C, between the normal colonies They were small (about 0 20 mm in diameter), grey, transparent, and composed of long fusiform, frequently distorted and poorly stained, filaments These microcolonies did not enlarge during the next days of incubation at 37° C nor at room temperature It was difficult to obtain subcultures from these colonies even if the media were enriched with serum or ascites The subcultivation was successful only if the cells were not too deformed and still viable In this case the subcultures behaved as normal ones Normal colonies and microcolonies could again be obtained from them

5 *Growth in Fluid Media*

Quick and typical changes from small coccoids to long fusiform cells occurred only on the surface of solid and semisolid media In fluid media the changes were not typical, except in the surface ring, especially when this remained isolated on the inner side of the glass tube after evaporation of water during a prolonged incubation period at 37° C

6 *Staining by the Gram and Feulgen's Technique*

Stained by the Gram method all coccoid and bacillary forms of both strains growing at the experimental temperatures mentioned above were uniformly Gram-negative The enlarged parts of the filaments showed a tendency to retain the violet stain The swellings in younger filaments were uniformly Gram-positive In the older filaments, and especially in their swellings, round and oval Gram-positive structures in otherwise Gram negative stroma were found

Stained by the Feulgen's method many Feulgen-positive uniform granules were observed in long filaments, especially in their swellings

7 *Effect of Factors other than Temperature on Size and Shape of Bacterium Anitratum*

The results obtained could be summarized as follows

A Physically and chemically deficient or adverse media prevent typical, by temperature induced, changes

B Aerobiosis is an indispensable condition in order that the temperature may act in the described way

C In strain P long and fusiform cells can be produced also at room temperature under the influence of penicillin, and, to a minor degree, under the influence of a low pH (5 8)

perature under the conditions described. The only exceptions were such secondary colonies as proved to be temporarily resistent to the temperature of 37° C.

In the development of these secondary colonies, an environmentally conditioned selection of mutants is evident. Resistent cells were grown through several generations with their distinctive character of biological behaviour, i. e., they could divide at 37° C as they did at room temperature. This type of changes is heritable, and as a rule, stable. However, they can revert in the case of reverse mutations. Appropriate conditions can in such cases reselect the original strain.

The results of the animal experiments are supposed to be in accordance with the in vitro experiences and, on the other hand, with the way of growth of the strains described in fluid media.

The relation between these forms and the stages of L type of growth should also be mentioned. L forms were discovered first by *Klieneberger-Nobel* (1935), and represent a specific, plastic, and pleomorphic but stable form of growth. (For further information the reader is referred to the extensive reviews by *Klieneberger-Nobel* (1951), *Dienes* (1951), and *Tulasne* (1951).) According to *Klieneberger-Nobel* (personal communication), the described forms can be regarded as transitional forms.

L forms display a special morphology of the colonies. The above described colonies of abnormally sensitive microorganisms correspond to the L type of colonies, being small, slow growing, and late in appearence. Fried egg like colonies were not found between the above described microcolonies. However, it has been shown recently that the typical L colony appearence depends on the physical conditions of media used (*Landman* 1958).

The large forms observed in the described strains of Bacterium anitratum are not interpreted as being due to some degeneration or involution phenomena nor to a regeneration cycle but simply to the blocked division in the continuously growing bacterial cell. Non dividing cells grow bigger and bigger, similarly to the cells of Tetrahymena pyriformis treated with intermittent heat shocks (*Scherbaum & Zeuthen* 1954). The final dimensions as compared with those in the primary cultures, however, are much bigger in Bacterium anitratum than in Tetrahymena pyriformis. Nevertheless, in their early stages, the late or never dividing monsters which develop under the influence of the deleterious temperature are still reversible in both organisms.

SUMMARY

Striking changes in the size and shape of Bacterium anitratum are demonstrated and found to be dependent on tem

media and found that none of these consistently produced abnormal morphological forms

The difference in size observed between microorganisms growing at low and high temperatures may be explained in the light of the above considerations Differences in form need another explanation

When cross wall formation is impaired, the cells begin to grow as long nonseptate filaments The fusiform and otherwise enlarged shapes are probably due to the disturbance in the formation of a rigid cell wall Weakened cell walls are then expanded by increased protoplasmatic pressure, and, probably, also because of impaired osmotic regulation

The mechanism underlying the weakening of the cell wall by high incubation temperature is not explained It remains unknown whether it happens through the interference of diaminopimelic acid which was found in the cell walls of bacteria and is known to play an important rôle in the synthesis of bacterial cell wall (*Weibull* 1958, *Pelzurra & Szybalsky* 1959), or whether some other important cell wall constituent is involved

It seems surprising that the temperature of 37° C could act so adversely on strain H which was isolated repeatedly from the human body The blocking effect on cell division of the temperature of ordinary incubation of pathogenic bacteria is understandable in the case of the contaminant P which is supposed to live freely in nature and to be adapted to lower temperatures In accordance with this consideration is the observation that the temperature which induced polymorphism in both strains had a more deleterious action on strain P Its survival time at 37° C was much shorter than the survival time of strain H at the same temperature Also the macroscopical growth of strain P was much poorer at 37° C than at room temperature These observations seem to indicate the degenerative nature of the bizarre forms

It is difficult to determine which phase of division was blocked by a temperature of 37° C From the form of most filaments it might be concluded that the adverse action of temperature became effective during the stage of cross wall formation or/and during the cell splitting stage The latter possibility seems to be indicated by the presence of some not completely segmented, filaments Nuclear growth and its division appear to be unimpaired This view is supported by many small Feulgen-positive granules found in the filaments The here described observations are in accordance with the view of *Tulasne* (1949) *viz* that the large cells continue normal functions of growth but have lost their ability to divide, and that the cytoplasmatic division is inhibited while nuclear division continues

The changes from small coccoids to large filaments were directly impressed by the temperature of 37° C These changes were not heritable The acquired character was lost promptly after the cells were grown in the absence of the inducing factor The changes were reversible at every subcultivation and at every transfer to the suitable tem-

FROM THE VIROLOGICAL DEPARTMENT OF THE MUNICIPAL LABORATORIES
OF GOTHENBURG AND THE DEPARTMENTS OF BACTERIOLOGY AND
PATHOLOGY I UNIVERSITY OF GOTHENBURG SWEDEN

SOME ASPECTS ON CULTIVATION OF TOXOPLASMA GONDII IN CELL CULTURES *

By

EBBA LUND, E LYCKE and P SOLRANDER

Received 10 ix 62

Toxoplasma gondii multiplies in a wide variety of cells From many points of view the use of cell cultures appears to be a suitable and direct method for studies of host cell parasite relationships Certain tissue culture systems allow observations in high power microscopy of living infected host cells (1) Thus they are especially useful when morphological criteria are to be applied

By means of tissue cultures sensitive and reproducible methods for quantitation of parasite growth may be obtainable In spite of an increasing literature information is still needed e g regarding the effect of the culture conditions on the growth of parasites The present report gives examples of variations in culture conditions affecting parasite growth

MATERIAL AND METHODS

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The RH strain of Toxoplasma gondii was used The parasites were obtained from freshly harvested peritoneal exudate containing 50 units per ml of heparin The strain was maintained in six week-old Swiss albino mice of own laboratory stock The exudate was harvested three days after the inoculation of the animals As a rule 0 3 0 6 ml of exudate per mouse was obtained and the pooled exudates from several mice were used

The antisera used were human sera which were positive in the dye test in dilution of at least 1 in 8000 The sera were inactivated at 56° C for 30 minutes As source of accessory factor fresh human sera were accepted if they were dye test negative and allowed a positive reference serum to react with a constant dye test titer in repeated tests

The cultures were inoculated with an exudate diluted 1 in 5 in tissue culture medium and incubated at 37° C for two hours The culture medium was then renewed and the cells carefully rinsed in order to avoid the toxic effect of the exudate on the cells

* This study was supported by a grant from Statens Medicinska Forskningsråd Sweden

ture The dependence on temperature of cell growth in the strains described is different from that of cell reproduction When besides division also growth of the organisms is inhibited, no fusiform shapes are found in spite of the, *otherwise in the described way acting incubation* temperature

Associate factors and conditions necessary in order to obtain typical forms, have been studied Aerobiosis and sufficient humidity of the media used are important co factors necessary in order to give rise to the typical changes

During prolonged incubation at 37° C, organisms, temporarily resistent to the adverse action of high temperature, develop They keep their form and small dimension in spite of their growth at 37° C

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The relation between the above mentioned and the L forms of bacteria is discussed

REFERENCES

Almquist F Studien uber das Verhalten einiger pathogener Mikroorganismen bei niedriger Temperatur Centralbl Bakt *48* 175 186 1909

Billing F Studies on a Soap Tolerant Organism a New Variety of Bacterium anitratum J Gen Microbiol *13* 252 260 1955

Dienes L & Weinberger H J The L forms of bacteria Bact Rev *15* 245 288 1951

Eisenstark A Ward C B Jr & Kyle T S A study of large bodies in Azotobacter agile J Bact *60* 525–531 1950

Hadley P The instability of bacterial species with special reference to active dissociation and transmissible autolysis J Infect Dis *40* 1–312 1927

Klieneberger Nobel F Filterable forms of bacteria Bacteriol Rev *15* 77 103 1951

Krogh A Thermostate und Thermoregulation Z Biol Technik u Methodik *3* Nr 4 5 1913

Landman O F Altenbern R A & Ginoza H S Quantitative conversion of cells and protoplasts of Proteus mirabilis and Escherichia coli to the L form J Bact *75* 567 576 1958

Lark K G & Maaloe O The induction of cellular and nuclear division in Salmonella typhi murium by means of temperature shifts Biochim et Biophys Acta *15* 345 356 1954

Lohnis F Studies upon the life cycle of the bacteria Mem Nat Acad Sci 16 2nd mem 1 252 1922

Olsen F Studies on the Intestinal Flora of Infants Thesis Kopenhagen 1949

Orskov J Some observations on aberrant bacterial morphology Acta path et microbiol scandinav 24 189 202 1947

Pulvurra M & Sybalski W Formation and multiplication of spheroplasts of E coli in the presence of Li chloride J Bact 77 614 620 1959

Tulasne R Cytology des Proteus et ses modifications sous l effect de la penicilline C R Soc Biol *143* 286 288 1949

Tulasne R Les formes L des bacteries Rev immun l *15* 223 251 1951

Schaub I G & Foley M K Diagnostic Bacteriology 4th edition The C V Mosby Comp St Louis 1952

Seeliger H Zur Systematik des Bacterium anitratum (Schaub und Hauber) Zentralbl Bakt I orig *159* 173 176 1953

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MATERIAL AND METHODS

HeLa cell cultures in milk dilution bottles roller tubes or Gey chambers (1) were used If not otherwise stated the medium consisted of 10 per cent dye test negative human or calf serum in Hanks balanced salt solution containing 0 5 per cent lactalbumin hydrolysate 100 IU of penicillin and 100 gamma of streptomycin per ml

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Hadley P The instability of bacterial species with special reference to active dissociation and transmissible autolysis J Infect Dis *40* 1 312 1927

Klieneberger Nobel E Filterable forms of bacteria Bacteriol Rev *15* 77–103 1951

Krogh A Thermostate und Thermoregulation / Biol Technik u Methodik *5* Nr 4 5 1913

Landman O E Altenbern R A & Ginoza H S Quantitative conversion of cells and protoplasts of Proteus mirabilis and Escherichia coli to the L form J Bact 75 567 576 1958

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Löhnis F Studies upon the life cycle of the bacteria Mem Nat Acad Sci 16 2nd mem 1 252 1922

Olsen E Studies on the Intestinal Flora of Infants Thesis Kopenhagen 1949

Orskov J Some observations on aberrant bacterial morphology Acta path et microbiol scandinav *24* 189 202 1947

Pitzurra M & Szybalski W Formation and multiplication of spheroplasts of E coli in the presence of Li chloride J Bact 77 614 620 1959

Tulasne R Cytology des Proteus et ses modifications sous l effect de la penicilline C R Soc Biol *143* 286 288 1949

Tulasne R Les formes L des bacteries Rev immunol *15* 223 251 1951

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Scherbaum O & *Zeuthen E* Induction of synchronous cell division in mass culture of Tetrahymena pyriformis Exp Cell Res *6* 221 227 1954

Scherbaum O & *Zeuthen F* Temperature induced synchronous division in the ciliate protozoan Tetrahymena pyriformis growing in synthetic and proteose peptone media Exp Cell Res Suppl *3* 312 325 1955

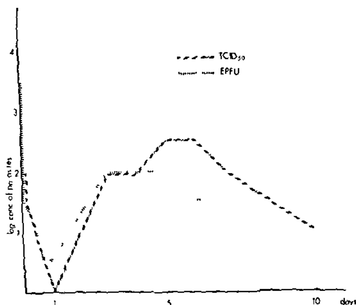


Fig 1

Rate of liberation of infective parasites from bottle cultures infected with 3 6 × 10^6 parasites per bottle The titrations of infectious units were carried out in tube cultures and registered as $TCID_{50}$ and on egg membranes as plaque forming units (EPFU) The log concentration of infectious units is plotted against time

TABLE 1

Titration of Toxoplasma gondii in Roller Tube Cultures

Log of exudate dilution	Average of daily microscopic readings of five tubes on day 1–4	5–9			10–12		
0	— — + +	+ + + + +	+ +	+ + +	+ + +	+ + +	+ + +
1	—	+ + + + —	+ +	+ + +	+ + +	+ + +	+ + +
2	+	+ + + + +	—	+	+	+	+
−3	— + —	— — — + —	+	+	—	+	+
4	—	— + — — —		—	—	—	—
5	— —	— — — — —	—	—	—	—	—
Control	— —	— — — — —	—	—	—	—	—

Growth of Parasites in Bottle Cultures

Bottle cultures were inoculated with parasites and incubated at 37° C for 10 days Samples were taken and the number of infective parasites was determined by titration in tube cultures and on chorioallantoic membranes of embryonated eggs Five bottle cultures with 10 ml of culture medium were used and each culture was inoculated with 3 6 millions of parasites The first sample was drawn directly after the inoculation and the second after one hour Fig 1 shows the results of

The medium was renewed every second or third day and the cultures were followed for varying lengths of time by daily observations in the microscope.

Titrations in roller tubes were performed by inoculation of 10 fold dilutions of the parasite containing fluid. Each dilution was inoculated into five tubes using 0.1 ml per tube. Cellular changes in the tube cultures were recorded as + (indicating any morphological changes not observed in non inoculated control cultures) ++ (degenerative changes in approximately 50 per cent of the culture) and +++ (the majority of the cells showing pathological changes).

Fifty per cent infectivity doses ($TCID_{50}$) per ml were calculated on results registered on day 12 as the highest dilution which caused at least + changes in half the number of inoculated cultures.

Titrations in bottle cultures were carried out by inoculation of 10 fold dilutions of parasite containing fluid. One ml of each dilution was inoculated in two bottles. The bottles were carefully rinsed after 3 hours at 37° C and the medium renewed. The number of clearly visible plaques appearing in the cell monolayer under liquid medium was counted. The result was given in tissue culture plaque forming units per ml.

When inoculated *Gey chambers* were used the intracellular parasites were registered in the following way: using phase contrast microscopy four groups of five cells each—if not otherwise stated—were selected at random in different parts of the culture. The number of infected and noninfected cells was counted and the different stages in parasite growth were registered.

The parasite divides soon after the penetration of the cell and subsequently the two daughter parasites divide as well. The parasites formed do not separate from each other before the host cell bursts but form a parasite clone. The different stages from the single penetrating parasite to the development of the large clone of numerous individuals are easily distinguishable in the microscope. The registration of the different stages was made in terms of infective units (IU). One IU may appear as one single parasite or any number of parasites in a clone derived from the one parent parasite which had penetrated the cell. Thus were registred the number of single parasites, i.e. parasites that have penetrated but not yet divided, paired parasites after the first division, clones containing four to sixteen parasites, clones with more than sixteen parasites and finally were recorded also the number of host cells which had bursted due to the parasite multiplication. The extracellular parasites were counted in a haemocytometer.

Titrations on egg membranes were performed using 0.1 ml inocula and 10 fold dilution steps. Each dilution was inoculated onto the artificially dropped chorioallantoic membranes of five embryonated eggs which had been preincubated for 12 days. The eggs were harvested 3 days after the inoculation. The number of clearly visible plaques was counted and the average number of plaques per ml was calculated.

EXPERIMENTS

Titration of Infective Units in Tube Cultures

Table 1 shows the recorded readings of a titration. The undiluted exudate contained one million parasites per ml. With magnifications of about 100 times the cellular changes in the tube cultures caused by the multiplication of parasites appeared in the microscope as uncharacteristic degenerations. They were first distinguishable as dark cytoplasmic granulations. Gradually scattered foci of completely degenerating cells were formed. In some cases a complete destruction of the cell culture followed. Often however the degeneration of the culture came to a standstill and did not proceed further during the period of observation. Only one per cent of counted parasites were recovered as $TCID_{50}$.

be given To some degree, however, it could be a result of the occurrence of multiple infected cells i e cells infected with more than one parasite A direct proportionality was found between the concentration of inoculated parasites and the percentages of the number of IU over the number of cells observed

TABLE 2

A Comparison between Different Methods for Titration of Infective Parasites The Recovery of Infectious Units per 100 Inoculated Parasites

Method	Number of units
Egg plaque forming units	0 5 — 2
Tissue culture infectivity doses $TCID_{50}$	1 — 2
Tissue culture plaque forming units	25 — 50
Infective units in Gey chamber cultures IU *	30 — 60

* See text

In Table 2 the results from different experiments are collected The number of parasites inoculated are compared with the number of infectious units when using different methods It seems that whereas 100 counted parasites represent only about one plaque forming unit on egg membranes and one $TCID_{50}$ they are equivalent to 25 50 plaque forming units in cell cultures and to 30 to 60 IU

Persistent Infection

In the experiments with titrations of Toxoplasma gondii in roller tube cultures it seemed as if the degenerative changes sometimes were inhibited The optical conditions in roller tube cultures do not permit high power microscopy and it was therefore attempted to study the 'inhibition more closely using cultures in Gey chambers The cultures were inoculated with different concentrations of parasites One group of cultures received a concentration of parasites resulting in infection of the majority of the cells To another group a lower concentration of parasites was added and only 10-20 per cent of the cells were infected after three hours

The heavily infected cultures degenerated totally within 4-5 days whereas cultures inoculated with the lower concentrations of parasites could be maintained for more than three months It seemed as if an equilibrium was obtained between the frequency of cell burst due o parasite multiplication and of mitoses of cells in the cultures This might explain why the development of degenerative changes seemingly may be inhibited

one such experiment In the figure mean values of log $TCID_{50}$ and of log number per ml of plaques on egg membranes are plotted against time in days

The methods for titration of parasites allowed demonstration of approximately one per cent of counted inoculated parasites One hour after the inoculation about 3 per cent infectious units remained in the tissue culture fluid The following day only a few infectious units were demonstrable suspended in the fluid On the third day after the inoculation multiplication and release of parasites into the medium occurred However, the number of infectious units were less than one tenth of the inoculated amount During the following three days there was obviously an equilibrium between the release of parasites into the medium and the loss of infectivity of the free parasites On the tenth day, when extended degeneration on the cell sheet was observed, the contents of infectious units in the fluid were very low The inactivation of parasites was thus not substituted any longer by parasite liberation

A Comparison between Different Methods for Titration of Infectious Parasites

Generally, when inoculation in eggs or roller tubes was used, about one per cent only of the counted parasites were demonstrable as infectious units Comparisons between different test systems were therefore carried out to see how much the number of infectious units found was depending on the test system used for titration

The infectivity of parasite suspensions were titrated on a) roller tube cultures, b) on cell monolayers of bottle cultures, c) on Gey chamber cultures and d) on chorio-allantoic membrane of eggs

Bottle cultures with cell monolayer were inoculated with dilutions of a parasite suspension Plaques were visible in the monolayers on the fourth day after the inoculation when large inocula were used (10^5 parasites or more per culture) but the plaques were almost confluent and could not be counted with accuracy After 12 days of incubation plaques could be counted in cultures inoculated with the more diluted parasite suspensions Then the ratio between the number of inoculated parasites and the number of plaque forming units observed was 5 0–2 5 The plaques were 1–2 mm in diameter and were visible even without staining with neutral red

The cell number in the Gey chamber cultures was estimated to be 2×10^5 to 5×10^5 per culture Parasites in concentrations varying from 600,000 to 20,000 were inoculated per culture and the number of IU relative to the number of cells observed was determined after one day of incubation Three hundred cells in each of the inoculated cultures were observed

Even with the highest concentrations of parasites used all cells of the cultures did not become infected The reasons for this cannot as yet

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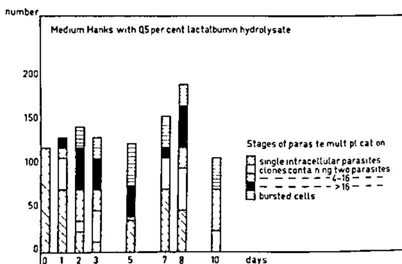


Fig 2

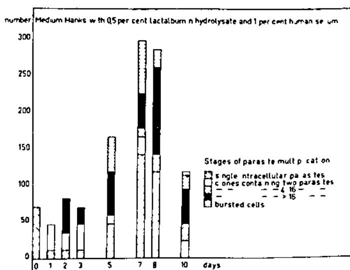


Fig 3

Growth of Parasites in Cells Cultivated in Different Media

HeLa cell cultures were grown for 48 hours in Gey chambers with a 10 per cent human dye-test negative serum The medium was then changed to one of the following media Hanks' solution (A), Hanks' solution with 0 5 per cent lactalbumin hydrolysate (B), medium B with addition of 1 per cent human dye-test negative serum (C), medium B with addition of 10 per cent serum (D), and finally medium B to which 20 per cent serum was added (E) Each medium was used on four cultures and three series of experiments were carried out

After inoculation of cultures with 120,000 parasites per culture the number of intracellular parasites and of clones of parasites were counted daily and recorded as the number of IU By the registration

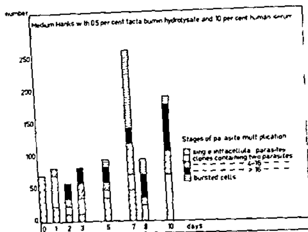


Fig 4

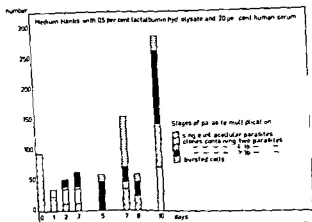


Fig 5

Figs 2–5

The rate of multiplication of intracellular parasites in cultures with different media The number of infective units (IU) cf text) at different stages of of development in 240 cells is plotted against time in days

of the different stages of parasite multiplication it was attempted to evaluate whether the culture conditions influenced the growth of the parasites

The results of the three series of experiments are summarized in Fig 2 5 For each type of medium (A-E) used, a total of 240 cells were observed In the cultures containing medium A only a few cells remained attached to the glass after the second day They were granulated and had fringed edges, and although many cells contained parasites they could not be maintained long enough to allow observations of parasite growth In Fig 2 the results are registered when medium

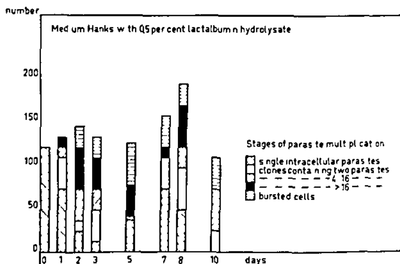


Fig 2

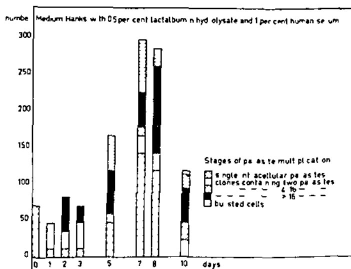


Fig 3

Growth of Parasites in Cells Cultivated in Different Media

HeLa cell cultures were grown for 48 hours in Gey chambers with a 10 per cent human dye-test negative serum The medium was then changed to one of the following media Hanks solution (A), Hanks solution with 0 5 per cent lactalbumin hydrolysate (B), medium B with addition of 1 per cent human dye-test negative serum (C), medium B with addition of 10 per cent serum (D), and finally medium B to which 20 per cent serum was added (E) Each medium was used on four cultures and three series of experiments were carried out

After inoculation of cultures with 120,000 parasites per culture the number of intracellular parasites and of clones of parasites were counted daily and recorded as the number of IU By the registration

the development of infection in a group of control cultures treated with normal serum

The number of cells in which infection was initiated in presence of antiserum was considerable smaller than the number of cells infected in the control cultures When infection was established, however, it proceeded with the same rate of multiplication in both types of cultures Finally an equilibrium was reached between the destruction of cells due to parasitization and the multiplication of uninfected cells

Growth of Parasites in Cultures of Different Cell numbers

Different dilutions of a HeLa cell suspension were used as seed for Gey chambers After two days of incubation each culture was inoculated with 200,000 parasites The cultures were observed daily, and the different stages of parasite multiplication were registered The average results of three such experiments are collected in Table 3 In each experiment was also included a group of 3 weeks old cultures treated as *mentioned above*

TABLE 3

Rate of Multiplication of Toxoplasma gondii in Cultures with Different Cell Numbers

Number of cells seeded	Number of cells per field of observation at time of inoculation	Time in days when 50 per cent of the IU appeared in the form of	
		clones containing 4 8 parasites	bursted cells
1×10^3	1	1	—†
5×10^3	4	1	1
10×10^3	6	1	2
25×10^3	20	1	7
50×10^3	25	1	7
—§	150	7	7

* See text

§ Three week old cultures were used

† Spontaneous degeneration of cultures occurred before bursts were observed

In cultures prepared by seeding 25,000–50,000 cells as well as in the 3 weeks old dense cultures, it took a week before half of the infected cells bursted while this happened already after 24 hours when 5,000 cells had been used as a seed

Fifty per cent of the IU were recorded as clones containing 4–8 parasites at 24 hours after the infection irrespectively of the number of cells seeded In the 3 week-old culture this stage was not reached, however, before one week after the inoculation

DISCUSSION

As Toxoplasma gondii is an obligate intracellular parasite the cell culture technique seems to be the most relevant method available for studies of the parasite host cell relationship Studies using whole orga-

B was used On day 0 the total number of parasites found intracellularly three hours after the inoculation are recorded Multiple infection, i e more than one parasite infecting one cell, occurred quite frequently, and the number of intracellular parasites is therefore not identical with the number of infected cells Readings on day 1 showed that about 50 per cent of the intracellular parasites had undergone at least one division On day 2 cell bursts due to parasite multiplication were observed and the majority of parasites had divided On day 5 and even more pronounced on day 7 an increase of single parasites was found indicating infection of cells with parasites produced in the culture On day 10 finally fewer infected cells were found and no single parasites were observed

Repeatedly, addition of serum to the medium was found to lower the number of intracellular parasites Fig 3 shows the results obtained when medium C was used In comparison with the results obtained with the serum free medium B only about half as many intracellular parasites were counted at three hours after the inoculation Moreover, the cycle of parasite reproduction seemed to be generally delayed with at least two days' delay of cell bursts On the other hand a marked increase in number of IU was demonstrable on day 7, so that the average number of IU exceeded one per cell Again, on day 10 a drop in the number of IU occurred

Increase of serum concentration to 10 per cent (D) and to 20 per cent (E) resulted in a further retardation in parasite multiplication (Figs 4 and 5)

The Addition of Specific Antibodies to the Culture Medium

To observe the effect of antibodies on an established infection a group of eight infected cell cultures was treated with antiserum-accessory factor mixtures 24 hours after the inoculation of the cultures with the parasites To another group of cultures, serving as controls, dye-test negative serum was added at the same time The final concentration of serum was 20 per cent

Antiserum did not inhibit the multiplication of the already intracellular parasites In all cultures clones were formed and cell bursts occurred at the same rate However, up to one week after the inoculation a difference in the ratio between infected and noninfected cells could be found in the two groups Twice as many cells remained uninfected in the antiserum treated cultures as in the cultures without addition of antiserum This quantitative difference disappeared gradually during the next week At about 14 days after the inoculation no difference was distinguishable

A group of eight cultures were pretreated with the mixture of antiserum and accessory factor 24 hours before the inoculation of the parasites and the progress of infection in these cultures was compared with

sites This might be explained by the shortness of the interval between the release of parasites from bursting cells and the penetration of these parasites into new host cells and by the rate at which the extracellular parasites are inactivated

In the present report it was mentioned that plaques are formed also under liquid medium i e without any agar overlay and that the efficiency of plating then seems to be of the same order of magnitude as reported by *Chaparas & Schlesinger* (3) The fact that distinct plaques are formed also on egg membranes indicates as well that the infection of a cell population is delimited to the cells penetrated by the inoculated parasites and is spread gradually to the cells in close contact with the primarily infected ones

Shimu u (4) has worked with stationary bottle cultures and found that to get a good yield one tenth of the cells in a culture should be infected and that if more cells were primarily infected the yield of parasites in the fluid increased very little

Schuhova (5) described persistent infections with toxoplasma of HeLa cells in stationary cultures In the present experiments it was found that if less than one half of the cells was infected primarily a steady state could be established with an equilibrium between the mitotic activity in the cell culture and cell destruction due to infection This seems the reason why in titration experiments (cf Fig 1) only heavily infected cultures are found to be destroyed completely while cultures receiving fewer parasites may continue to contain only restricted areas of infected cells In the present experiments it was found that presence of antibody and accessory factor did not affect an established infection in a culture On the contrary a steady state identical with the just described persistent infection of a culture was established The spread of parasites from cell to cell occurred obviously without antibody contact sufficient for the inactivation of the *parasites*

While certain observations seem explainable by properties of the parasites the type of cultured cells used have also been reported to influence the rate of multiplication *Cook & Jacobs* (6) have reviewed the literature on growth of toxoplasma in tissue culture They have studied parasite growth in different cell lines cultured in roller tubes and found that the rate of cell degeneration caused by the parasite varied with the cell type used but that the rate of penetration was independent of the cell type *Hogan Yoneda & Zweigart* (2) also found variations in the rate of multiplication in different cell lines as judged from the rate of formation of rosettes and cysts We found that cultures of human embryonic kidney cells allowed faster multiplication of the parasites than HeLa cultures When cultures of the same age and with approximately the same cell number were compared the age and cell number seemed more important for the rate of multiplication than the type of cells used *Shimu u* (4) gives examples of how the cell number and the age of cell cultures affect the number of parasites released

nisms, *e g* mice or rabbits, will always be complicated by many factors which can be excluded in cell cultures Even in cell cultures many variables are present, however They are principally connected with the tissue culture method used, the type of host cells and the source of parasites It may be of interest to reconsider the consequences of previous results (1) concerning the liberation of toxoplasma from the host cells before the results from different cell culture methods are compared It was found that the parasites, which were released through the bursting of a host cell, were actively moving and rapidly, often within few seconds, penetrated into neighbouring cells Whereas the primarily infected host cells were infected with one or a few parasites these secondarily infected cells were invaded by several parasites so that the cytoplasma might become completely filled with parasites Each parasite gave rise to a clone The bursting of the massively invaded host cells occurred after very few divisions of the parasites Thus the number of parasites produced in this secondary multiplication cycle was relatively small If the culture was kept for longer periods and new cells were still available the infection pattern described for the secondarily infected cells was repeated

If the parasites did not gain entrance into new cells very soon the chance that they would get in at all was diminished as they lost their motility Therefore, also very few parasites penetrated into the cells if the cells were located on the upper side of a stationary culture *Hogan, Yoneda &Zweigart* (2) who worked with Leighton cultures found that the organisms seemed to spread in the immediate neighbourhood and that cells which were remote from the original zone of infection remained uninfected The results of *Chaparas & Schlesinger* (3), who worked with a plaque counting technique, confirm the importance of sedimentation for obtaining adsorption and penetration It was shown that increased viscosity of the medium diminished the number of plaque forming units absorbed in a certain time, while it was increased by centrifugation of the culture

As long as a sufficient number of susceptible cells was in the immediate neighbourhood the newly liberated parasites rapidly penetrated into new cells and very few infective parasites remained extracellularly As the extracellular parasites soon become inactivated spread of infection in the culture is inhibited Subsequently the concentration of parasites in the tissue culture medium remains low

When these observations are taken into consideration it seems possible to understand certain experimental results somewhat better *Chaparas & Schlesinger* (3) have reported that they were not able to obtain a release into the medium of more than an average of two parasites per cell although the cells lining the plaques usually contained a large number of organisms In the present study the maximal concentration of parasites released into the medium fluid of inoculated bottle cultures never exceeded one tenth of the inoculated number of para-

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HI ANTIBODIES IN FERRETS CROSS-INFECTED WITH DISTANTLY RELATED INFLUENZA VIRUSES

By

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Received 20 ix 62

The author previously showed that when ferrets had been infected with influenza A_2-virus, an infection some months later with Dutch 56 A_1-virus or swine influenza virus could produce a substantial increase of the haemagglutination inhibiting (HI) A_2-antibody titre, while the other A-strains produced only a moderate or no recall (5,6). However, only one ferret had been tried for each of the other A-strains, and it was therefore decided to repeat the experiments with several animals in order to learn if Dutch '56 A_1 or swine virus might be more related to the A_2-strains than the other A-strains are.

Examined in the present investigation were also the heterologous antibody recall between the Dutch '56 A_1-type and the A type isolated from horses, a recall observed by *Masurel & Mulder* (18), and the recall between A and B strains which was observed by the author (5,7). – *In another study* described in the present paper ferrets were cross infected with a series of A-strains which were administered one after another at intervals of several months.

MATERIALS AND METHODS

Viruses. A/Swine 15 ... *Masurel.* It had been ... 2?7 mouse passages. Be... HI test in addition to ... Two lines of ... numerous ...

... strains and the horse influenza virus ... Prague 56) employed have been given earlier (10 12).

Sera. In the experiments *described* in Sections 1, 2 and 3 the bloodsamples were drawn by means of heart puncture on the inoculation days, about 12 days after the

The author is indebted to Mrs. *G. Hoe* and Mr. *P. Selberg* for skilled technical assistance.

Cherning & Weller (7) and *Shimizu* (4) have shown the effect of renewal of the tissue culture medium on the rate of multiplication as measured by the number of parasites released It was also found (4) that the serum content in the culture medium influenced the rate of release of the parasites so that higher serum concentration caused faster release of parasites In the present work it was found that increased serum concentration slowed down the rate of multiplication of the parasites It cannot be settled from these experiments in which way the parasite growth was influenced by the culture medium The serum concentration of the medium might have influenced the growth and general, metabolic activity of the cell cultures and consequently the serum concentration might also affect the rate of multiplication of parasites

The method of titrating infective parasite by registration of IU in Gey chambers has been found to be as sensitive as determination of plaque forming units It gives results already on the day following inoculation and it allows not only an estimation of the number of infectious units but also gives possibilities for direct determinations of the rate of multiplication When working with this system it has been found, however, that far going standardization of the cell cultures and of the source of parasites is necessary to make full use of its potential possibilities The method will be described in detail in a following report

SUMMARY

The infectivety of Toxoplasma gondii was assayed in different tissue culture systems and in embryonated eggs About one per cent of inoculated parasites could be demonstrated as infective parasites by means of eggs or HeLa cell roller tube cultures Using a plaque technique or a method by which infective parasites were demonstrated directly as intracellular parasites 25 to 60 per cent of the inoculated parasites were recovered Culture conditions such as the serum concentration, the presence of antibody or the number of cells in the culture influenced the progress of infection

REFERENCES

1 *Lund Ebba Lycke E & Sourander P* A cinematografic study of Toxoplasma gondii in cell cultures Brit J of Exp Path XLII 357 1961
2 *Hogan J H Yoneda Ch & Zweigart Phyllis* Growth of Toxoplasma strains in … Am J of Ophtalmology 51 920 1961
3 … assay of Toxoplasma on monolayers … Exp Biol & Med *102* 431 1959
4 Shimizu K … gondii Japanese J of Veterinary Science 23 33 1961
5 *Schuhova Vera* Langfristige kulturen des Toxoplasma gondii in HeLa Zellen Zentralbl f Bakt Parasit und Infekt Orig *168* 631 1957
6 *Cook M Katherine & Jacobs J* Cultivation of Toxoplasma gondii in tissue culture … J of Parasit 44 172 1958
7 *Chernin E &* … the growth of Toxoplasma gondii … unate tissues J of Parasit 43 33 1957

seen that the cross infection frequently resulted in an A_2-titre increase However, only with the Dutch '56 A_1-strains this increase was 4 fold or greater, namely in the 5 animals cross infected with A_1/Netherlands 36 56 in 4 of the 5 animals cross infected with A_1/Denmark/2 57 and in 1 of the 5 animals cross infected with A_1/Denver/1/57 The A_2-titre maximum was reached earlier than the titre maximum to the cross infecting strain

In these titrations duck allantoic grown A_2-virus instead of chick allantoic grown had been employed in order to avoid any influence on the titres by the antibody to chick material (9) However, the titres of the virus specific antibodies were so high that this precaution actually was unnecessary

Similar cross infection experiments as the one above were performed on 2 ferrets with A_2/Japan 305 57 followed by PR 8, on 2 ferrets with A_2Singapore/1/57 x A/Equi Prague/56, and on 2 ferrets with A_2Singapore/1/57 x A/Fowl plague In none of these animals as much as a 4-fold anamnestic A_2-titre rise was provoked

2 *Horse Influenza Virus Infecting before or after Dutch '56 A_1-strains*

In this experiment A/Equi Prague 56 was given before or after different Dutch '56 A_1 strains It is known to be no direct cross-inhibition between these two types Infection took place by intranasal instillation of chick allantoic fluid The results are presented in Table 2 It is seen that both virus types are able to provoke an anamnestic titre rise to the other one, though in some of the ferrets the rise is small (less than 4 fold) or absent

TABLE 2

Titre Changes of the Haemagglutination Inhibiting Antibodies to the first of two Cross Infecting Viruses in 6 Ferrets Given the Horse Influenza Strain A/Equi/Prague/56 before or after a Dutch 56 A_1 strain The Ratios Are Shown see Table 1

Ferret cross-infected with		Days between the two inoculations	Titre decrease of antibody to the first virus between the two inoculations (fold)	Titre increase of antibody to the first virus 10 days after the cross infection (fold)
A Equi Prague/56 A_1 Denmark/2 57	and	306	4	10
A/Equi Prague/56 A_1/Denmark/2 57	and	269	3	no change
A Equi Prague/56 A_1 Denver/1/57	and	269	2	4
A_1 Denmark/2 57 A/Equi Prague/56	and	222	6	4
A_1 Denver 1/57 A Equi Prague/56	and	222	16	2
A_1/Netherlands/36/56 A Equi Prague 56	and	306	6	2

first inoculation and 10, 20 and 30 days after the second inoculation. In Section 4 usually one or two samples were drawn after each inoculation.—The animals were inoculated by intranasal instillation of 1 ml of virus bearing chick allantoic fluid (in a single experiment duck all. fl.). Inoculations and heart punctures were usually performed without ether anaesthesia because the intensive smell of ether seemed to be a greater nuisance to the animals than the sensation of the intranasal instillation of allantoic fluid, and of the quickly performed heart puncture.

Haemagglutination inhibition (HI) test. The sera were pretreated with cholera filtrate (22) and absorbed with fowl red cells and the HI test performed as previously described (7, 12, 17).

Antibody absorptions were performed with concentrated virus suspensions and subsequent high speed centrifugation according to a method described previously (11).

RESULTS

1. *Cross-Infections with A_2 Followed by Different A-Strains*

In the following experiment the ferrets were first infected by intranasal instillation of A_2/Japan/305/57 EFML, and later with another A-strain. All animals were infected with A_2-virus from the same batch of chick allantoic fluid, stored at —65° C. The interval between the two inoculations was 5–6 months. Also the cross-infection took place by intranasal instillation of virus bearing, chick allantoic fluid. The sera were tested with A_2/Japan/305/57 EFML and the cross infecting strain concerned.

TABLE 1

Titre Changes of the Haemagglutination Inhibiting A_2 Antibodies in 46 Cross Infected Ferrets

Infected with A₂/Japan/305/57 EFML followed by (either or)	Number of days between the infections	A₂-antibody titre decrease before and increase after the cross infection (fold) Ferret No. 1		2		3		4		5	
A/Swine/1976	155	16	3	4	3	16	2	16	n.c.	4	n.c.
WS	154	12	3	6	2	4	2	8	n.c.	4	n.c.
Mel	160	12	n.c.	8	n.c.						
Inota	155	8	2	12	n.c.	6	n.c.	4	n.c.	n.c.	n.c.
FM 1	154	32	2	24	2	16	2	6	2	4	n.c.
A_1/Sweden/3/50	176	6	n.c.	3	n.c.	2	n.c.	2	n.c.		
A_1/Eire/17/55	184	6	n.c.	3	n.c.	2	n.c.	2	n.c.	n.c.	n.c.
A_1/Netherlands/36/56	176	*4*	*16*	*2*	*8*	*8*	*4*	*4*	*4*	*3*	*4*
A_1/Denver/1/57	156	*16*	*48*	12	2	8	2	8	n.c.	8	n.c.
A_1/Denmark/2/57	178	4	*16*	3	*16*	*6*	*12*	*6*	*6*	6	n.c.

The *first* of the two values is the ratio between the titre on the 12th day after the first infection and the titre on the day of the cross infection. The second value is the the ratio between the titre 10 days after and on the day of the cross infection. n.c. means: no change.

Table 1 shows the A_2-antibody titre changes resulting from the cross-infection. In addition the decline of the A_2-antibody titres during the long interval prior to the cross infection has been tabulated, showing that regularly a heavy reduction took place during this interval. It is

seen that the cross infection frequently resulted in an A_2-titre increase However only with the Dutch 56 A_1-strains this increase was 4 fold or greater namely in the 5 animals cross infected with A_1/Netherlands 36/56 in 4 of the 5 animals cross infected with A_1/Denmark 2 57 and in 1 of the 5 animals cross infected with A_1/Denver/1/57 The A_2-titre maximum was reached earlier than the titre maximum to the cross infecting strain

In these titrations duck allantoic grown A_2-virus instead of chick allantoic grown had been employed in order to avoid any influence on the titres by the antibody to chick material (9) However the titres of the virus specific antibodies were so high that this precaution actually was unnecessary

Similar cross infection experiments as the one above were performed on 2 ferrets with A_2 Japan 305 57 followed by PR 8 on 2 ferrets with A_2 Singapore/1/57 x A/Equi Prague 56 and on 2 ferrets with A_2 Singapore/1/57 x A Fowl plague In none of these animals as much as a 4 fold anamnestic A_2-titre rise was provoked

2 *Horse Influenza Virus Infecting before or after Dutch 56 A_1 strains*

In this experiment A/Equi Prague 56 was given before or after different Dutch 56 A_1 strains It is known to be no direct cross inhibition between these two types Infection took place by intranasal instillation of chick allantoic fluid The results are presented in Table 2 It is seen that both virus types are able to provoke an anamnestic titre rise to the other one though in some of the ferrets the rise is small (less than 4 fold) or absent

TABLE 2

Titre Changes of the Haemagglutination Inhibiting Antibodies to the first of two Cross Infecting Viruses in 6 Ferrets Given the Horse Influenza Strain A/Equi/Prague/56 before or after a Dutch 56 A_1 strain The Ratios Are Shown see Table 1

Ferret cross-infected with		Days between the two inoculations	Titre decrease of antibody to the first virus between the two inoculations fold	Titre increase of antibody to the first virus 10 days after the cross infection (fold)
A/Equi Prague 56 A_1/Denmark/2 57	and	306	4	10
A/Equi Prague/56 A_1 Denmark/2 57	and	269	3	no change
A/Equi Prague/56 A_1 Denver 1 57	and	2[illegible]3	2	4
A_1 Denmark 57 A/Equi Prague 56	and	23[illegible]	6	4
A_1 Denver 1/57 A Equi Prague/56	and	222	16	2
A_1/Netherlands/36 56 A Equi Prague 56	and	306	6	2

Thus the Dutch '56 A_1-type has presented cross-reaction with A/Equi as well as with A_2. However, ferrets cross-infected with the latter two viruses, namely 3 animals with A_2 × A/Equi and 2 with A/Equi × A_2, failed to reveal any development of Dutch '56 A_1-antibody, neither was there as much as a 4-fold, heterologous, anamnestic increase.

When sera from the A/Equi × A_1/Denver ferret were absorbed with A_1/Netherlands virus, a distinct reduction of the A/Equi antibody titre was recorded after the cross-infection, but not before, showing that the cross-infection had given rise to antibodies of double (A/Equi + A_1) specificity. The heterotypic antibody increase provoked by the cross-infection in this animal could therefore hardly be ascribed to any cryptic contamination of the A_1/Denver virus with A/Equi material. – An attempt to absorb A/Equi antibody in the first one of the two A/Equi × A_1/Denmark ferrets, and in the A_1/Denmark × A/Equi ferret by treatment with the same suspension of A_1/Netherlands virus as above, failed. That cross-reacting antibody could be demonstrated only occasionally, confirmed the impression that the relationship between the two types must be a fairly distant one.

3. *The B-Strain Lee Infecting before or after the A-Strain PR-8*

14 ferrets were given Lee followed 5½ months later by PR-8. Both PR-8 lines (the "egg-line" and the "mouse-line") were used in order to reduce the possibility of faulty conclusions due to any contamination with influenza B-virus. The purity of the two PR-8 inocula was checked by inoculating eggs with the allantoic fluids incubated with serial dilutions of PR-8 convalescent, heat inactivated ferret serum. As a precaution a serum was used which had been received from another institute. Only PR-8 was recovered from the eggs.

TABLE 3

Titre Changes of the Haemagglutination Inhibiting Lee-Antibodies in 14 Ferrets Infected with the Influenza B Strain Lee Followed by the Influenza A Strain PR 8 of which two Different Lines Were Employed. The Ratios Are Shown, see Table 1

Infected with Lee followed by (either or)	Lee antibody titre decrease before and increase after the PR-8 infection (fold) — Ferret No. 1		2		3		4	
PR 8 egg line dripped intranasally	6	4	3	2	6	n.c.	4	n.c.
PR 8 egg line naturally transmitted	6	n.c.	3	n.c.	n.c.	n.c.		
PR 8 mouse line dripped intranasally	32	3	8	n.c.	6	n.c.	4	n.c.
PR 8 mouse line naturally transmitted	6	4	16	2	32	n.c.		

The 14 ferrets were given the Lee-virus as infected duck allantoic fluid by intranasal instillation. 8 of these animals were cross-infected with PR-8 by intranasal instillation of infected chick allantoic fluids

The remaining 6 animals were kept together with their inoculated cage mates the whole time, these 6 animals too became cross infected with PR 8, as revealed by antibody development Whether these 6 "naturally" infected ferrets contracted the PR 8 infection through chick allantoic particles or through virus particles which had grown in the inoculated cagemates, is unknown and hardly essential

Various antigens were employed in the titration of the Lee antibody content Virus harvested from mouse lungs was used in order to avoid the activity of antibody to host material Heated chick allantoic fluid virus was used in an attempt to detect any residual normal inhibitor activity, but no such activity was observed There was a good agreement between the various antigens as regards the titre changes recorded, only twice out of 125 times as much as a 2-fold difference between the parallels was noted The results obtained with a mouse grown antigen are presented in Table 3 It is seen that in 2 ferrets a 4 fold. anamnestic Lee titre increase has been produced Table 4 shows the titres recorded in these two animals As regards the number of "days after infection with PR-8" in the socalled "naturally" infected ferret, it was arbitrarily assumed that the animal caught the infection on the day when the inoculated cagemates were dripped, although it might as well have happened a few days later – A serologic difference between the two lines of PR-8 is noted

TABLE 4

Heterotypic Anamnestic Antibody Titre Increase in two Cross Infected Ferrets see Table 3

	Days after infection		Haemagglutination inhibition titres when tested with				
	with Lee	with PR-8	Chick grown Lee	Heated chick grown Lee	Mouse grown Lee	PR-8 egg line	PR-8 mouse line
No 1 of the ferrets infected with Lee followed by PR 8 egg line dripped intranasally	0		<30	<40	<40	<20	<20
	11		960	1920	640	<20	<20
	163	0	180	280	120	<20	<20
	173	10	720	1120	480	1280	320
	183	20	240	320	240	1280	400
	199	36	240	280	160	640	320
infected with Lee followed No 1 of the ferrets infected with Lee followed by PR 8 mouse line transmitted naturally	0		<30	<40	<40	<20	<20
	12		1920	3840	1280	<20	<20
	164	0	180	280	240	<20	<20
	174	10	960	1120	960	160	320
	184	20	480	960	640	640	2560
	200	36	240	480	240	240	640

Only 2 ferrets were given PR-8 followed by Lee, the interval was 10 months, and during this the PR-8 antibody titre fell from 2,000 to 200, resp 100 Both animals received PR 8 by intranasal instillation of chick allantoic fluid, and one of them Lee in the same way, this

TABLE 5

Haemagglutination Inhibition Test on Sera from Ferrets Cross Infected with a Series of Influenza A Virus Strains

Day when the blood sample was drawn and the eventual inoculation performed	Influenza virus strain used for inoculation	Titre of haemagglutination inhibiting antibodies against									
		A Swine 1976		PR 8		FM 1		A_1 Netherlands 36/56		A_2 Japan 305/57 FM1	
		Ferret No 109	Ferret No 110	Ferret No 109	Ferret No 110	Ferret No 109	Ferret No 110	Ferret No 109	Ferret No 110	Ferret No 109	Ferret No 110
June 10 1959	A/Swine/1976	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9
June 24 1959		432	144	<9	<9	<9	<9	<9	<9	<9	<9
Febr 19 1960	PR 8	36	36	<9	<9	<9	<9	<9	<9	<9	<9
March 2 1960		432	144	864	432	72	<9	<9	<9	<9	<9
May 13 1961	FM 1	45	72	45	31	<9	<11	<9	<11	<9	<11
May 24 1961		108	576	108	144	36	72	<9	<9	<9	<9
Nov 23 1961	A_1/Netherlands/36/56	36		18		<9		<9		<9	
Dec 4 1961		288		36		96		12		<12	
May 18 1962	A_2/Japan/305/57 FMI	72		36		<9		<9		<9	
May 28 1962		288		36		9		<9		72	
June 7 1962		216		36		<9		<9		144	

TABLE 5 (cont.)

Day when the HI sample was drawn and the eventual inoculation performed	Influenza virus strain used for inoculation	Titre of haemagglutination inhibiting antibodies against									
		A Swine 15 MI		PR 8		FM 1		A_1 Netherlands 36/56		A_2 Japan 305/57 FLMP	
		Ferret No 112	Ferret No 111	Ferret No 112	Ferret No 111	Ferret No 112	Ferret No 111	Ferret No 112	Ferret No 111	Ferret No 112	Ferret No 111
June 10 1959	A_1/Netherlands/36/56	<9	<9	<9	<9	<9	<9	<9	<9	<9	<18
June 24 1959		<9	<9	9	<9	<9	<9	4608	864	<9	<18
Febr 19 1960	FM 1	<9	<9	<9	<9	<9	<9	108	36	<9	<18
March 2 1960		108	18	576	216	864	216	4608	2304	<9	<18
May 13 1961	PR 8	<9		27		36		432		<9	
May 23 1961		36		2304		864		4608		<9	
Nov 23 1961	A/Swine/1976	<18		108		72		576		<9	
Dec 4 1961		<18		108		144		576		<9	
May 18 1962	A_2/Japan/305/57 FLMP	<18		108		72		576		<9	
May 28 1962		<18		108		72		1152		288	
June 7 1962		<18		108		72		1152		864	

TABLE 5

Haemagglutination Inhibition Test on Sera from Ferrets Cross Infected with a Series of Influenza A Virus Strains

Day when the bloodsample was drawn and the eventual inoculation performed	Influenza virus strain used for inoculation	Titre of haemagglutination inhibiting antibodies against									
		A Swine 1976		PR 8		FM 1		A_1 Netherlands/36/56		A_2 Japan 305/57 FMI	
		Ferret No 109	Ferret No 110	Ferret No 109	Ferret No 110	Ferret No 109	Ferret No 110	Ferret No 109	Ferret No 110	Ferret No 109	Ferret No 110
June 10 1959	A/Swine/1976	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9
June 24 1959		432	144	<9	<9	<9	<9	<9	<9	<9	<9
Febr 19 1960	PR 8	36	36	<9	<9	<9	<9	<9	<9	<9	<9
March 2 1960		432	144	864	432	72	<9	<9	<9	<9	<9
May 13 1961	FM 1	45	72	45	31	<9	<11	<9	<11	<9	<11
May 23 1961		108	576	108	144	36	72	<9	<9	<9	<9
Nov 23 1961	A_1/Netherlands/36/56	36		18		<9		<9		<9	
Dec 4 1961		288		36		96		12		<12	
May 18 1962	A_2/Japan/305/57 FMI	72		36		<9		<9		<9	
May 28 1962		288		36		9		<9		72	
June 7 1962		216		36		<9		<9		144	

DISCUSSION

The results of the cross-infection experiments described in this and two previous papers by the author (5,6) agree with those obtained in similar experiments by *Masurel & Mulder* (18), and in human vaccination experiments reported by *Mulder* (19) In all these investigations the Dutch '56 A_1 strains seem more capable than the other human A-strains to provoke an anamnestic A_2-titre rise With the swine influenza strain the results were less convincing in this respect, as none of the five $A_2 \times$ swine virus ferrets in the present investigation developed as much as a 4 fold, anamnestic A_2-titre rise *Masurel & Mulder* (18) found that 9 of 16 ferrets cross infected with A_2 followed by a Dutch '56 A_1-strain presented at least a 4-fold, anamnestic A_2-titre rise, while an A_1-strain from 1949 gave this rise in 2 of 8 animals, PR 8 in none of 8, and swine virus in 3 of 9 *Mulder* (19) found that the percentage frequency of the reinforcement of recently developed A_2-antibody was 20 per cent due to Dutch '56 A_1 vaccine, 3 per cent due to PR-8 vaccine, and 7 per cent due to swine virus vaccine It may be inferred from these different investigations that there is more A_2-antigen (or A_2-like antigens) in the Dutch '56 A_1 than in the other human A-strains The apparently closer relationship between the Dutch '56 A_1 and the A_2-virus combined with the fact that former was succeeded by the latter suggests that this A_1-virus may be the origin of the A_2-type

A study of strain relationship based on heterologous recall in sera from A_2-patients is more difficult because of the mixed, partly unknown antigenic experience (14, 18, 21)

As regards the complement fixing antibodies against soluble antigens they were not tested in the present investigation In another study (8) paired sera from influenza B patients were tested with soluble A-antigen and found to have a slight tendency to increase On the other hand no tendency of the B titres to increase was found in an analogous examination of influenza A patients

In a recent investigation (7) the author demonstrated that a cross infection with A_2-strains was able to increase the antibody titre to previously infecting influenza B [illegible] us In the present investi- [illegible] th the A_0-strain PR 8 was [illegible] months previously infecting B strain Lee, in 2 of the 14 ferrets this increase was very distinct (4 fold) It has thus been demonstrated that A_0 as well as A_2-virus are capable of provoking an anamnestic antibody increase to influenza strains which do not belong to the A-group The author sees no reason why anamnestic HI increases between myxovirus strains having different soluble antigens should be confined to the strains studied in the experiments mentioned above

The cross infections described in Section 4 of this paper frequently resulted in high titred antibodies to strains which had not been em

animal transmitted the Lee-infection to the other one In none of the animals the cross-infection with Lee produced as much as a 2-fold PR 8 titre increase

4 *Serial and Simple Cross-Infections with Pre-Asian A-strains*

The serial cross-infections were performed mainly to see if A_2 antibody might appear A positive result would throw light on the existence of pre-pandemic A_2-antibody observed in older people by *Mulder & Masurel* (20) and confirmed by other workers (3,4) In the complement fixation test with elementary bodies it had been found that infections with pre-Asian strains could produce A_2-antibody (13, 15)

The ferrets were cross infected at comparatively long intervals (several months) in order to make the experimental conditions analogous to those found in spontaneous, human infections *Masurel & Mulder* (18) performed similar experiments, except that the intervals were only 3 weeks *Bartolomei Corsi* (2) cross-immunized mice None of these workers were able to produce A_2-antibodies in this way The attempts in the present investigation were also negative As a precaution the sera were tested with duck allantoic grown viruses in order to exclude haemagglutination inhibition by antibody to host material Only A/Swine-15EM was not available as duck grown when needed for the test, and was instead employed as chick grown in the presence of normal, cholera treated allantoic fluid which was known to neutralize host specific antibodies (9) The results are shown in Table 5 It is seen that until the A_2-infection had taken place, no A_2-antibodies had appeared Neither did the A_2-antibody response to the A_2-infection seem to be accelerated, it was found in repeated titrations that the A_2-titres of the 20 day-samples were higher than those of the 10-day-samples

As regards the relationship between the pre-Asian strains, it was noted that in ferret No 109 (though not in No 110) swine virus followed by PR-8 produced FM-1 antibody In ferrets No 111 and 112 A_1/Netherlands followed by FM-1 gave antibodies to PR-8 and to swine virus The latter was detected only by means of the broadly reactive virus A/Swine-15 ME – None of the 4 ferrets developed antibody to horse influenza virus Neither did 5 ferrets infected with the horse virus developed antibodies to any of the other strains mentioned above

In an additional experiment two ferrets received FM-1 followed by A_1/Netherlands, and this cross-infection produced high-titred antibodies to swine influenza virus and to PR-8 The same phenomenon showed two ferrets which received A_1/Denver/1/57 after the FM 1 infection A ferret which received PR-8 followed by FM-1 presented after the cross-infection antibody to swine virus, and a ferret which had received FM-1 followed by PR 8 presented antibodies to swine virus and to A_1/Netherlands In none of the animals A_2-antibody was detected The interval between the infections was about half a year

8 *Harboe A* : Unpublished

9 *Harboe A, Borthne B & Berg K* Antibody against normal egg material resulting from influenza vaccination Acta path et microbiol scandinav 53 95 103 1961

10 *Harboe A & Reenaas R* The course of antibody development to influenza A_2 strains Examined in the haemagglutination inhibition test with viruses of different sensitivity to homologous antibody Acta path et microbiol scandinav 46 266–272 1959

11 *Harboe A & Reenaas R* Antibody crossabsorptions between influenza A_2 strains Acta path et microbiol scandinav 48 133 139 1960

12 *Harboe A Reenaas R & Juul K* Influenza A_2 strains against A and A_1 strains

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14

15

16 *Isaacs A* Antibodies to influenza viruses in the sera of Nigerians J Hyg 55 485 488 1957

17 *Isaacs A Gledhill A W & Andrewes C H* Influenza A viruses Laboratory studies with special reference to European outbreak of 1950–1 Bull World Health Organ 6 287 315 1952

18 *Masurel N & Mulder J* Studies on the content of haemagglutination inhibiting antibody for swine influenza virus A in sera from people living in the Netherlands in 1957 1958 Verhandel Nederlands Inst Praevent Gen No 52 Leiden 1962

19 *Mulder J* Communication at the Boerhaave Course on Respiratory Virus Diseases Leiden 1962

20 *Mulder J & Masurel N* Pre-epidemic antibody against 1957 strain of Asiatic influenza in serum of older people living in the Netherlands Lancet i 810–814 1958

21 *Nelson M & Lewis F A* A relationship between swine and Asian strains of infl

22 *Veen J*
fl
te

23 *Zhdanov V M & Stakhanova V M* A study of human sera for antihaemagglutinins to influenza like animal viruses Acta virol 4 187 188 1960

ployed for the infections, and which showed little or no cross-inhibition by antisera to each of the infecting strains However, A_2-antibodies never appeared as a result of such *cross-infections, they appeared only* as a result of an A_2-infection, and the latter produced no other antibodies Horse influenza virus behaved in the same way As regards human sera, *Zhdanov* (23) found no antibody to the latter The findings confirm that the A_2-strains belong to one antigenic grouping, the other human A-strains and swine influenza virus to another, while horse influenza virus can be considered as a third (18) The appearance of swine influenza antibody in ferrets cross-infected with A_1-strains furnishes an explanation to the observation of this antibody in some younger people (16)

SUMMARY

1 When groups of ferrets which had been infected with influenza A_2-strains were cross-infected half a year later with different other A-strains, the Dutch '56 A_1-strains produced a greater anamnestic A_2-titre rise than the other strains did

2 It was confirmed that in cross-infected ferrets Dutch '56 A_1 and horse influenza virus could produce a great, anamnestic antibody increase to each other After cross-infection cross-absorbing antibody could be demonstrated

3 Cross-infection with the A-strain PR-8 produced a 4-fold, anamnestic antibody increase to the B strain Lee in 2 of 14 ferrets which had been infected with Lee half a year before

4 A_2 and horse influenza antibodies failed to appear in ferrets which had been cross-infected with a chain of A-strains, which were administered at intervals of several months between each strain – Swine influenza antibody appeared after cross-infection with PR-8 and FM-1, and also with FM-1 and Dutch '56 A_1-strains

REFERENCES

1 *Barb K Farkas E Romvary & Takatsy Gy* Comparative study of influenza virus strains isolated from domestic animals in Hungary Acta virol 6 207 213 1962

2 *Bartolomei Corsi O* Rapporti immunologici fra virus influenzali A_2 e virus dei tipi suino A A_1 Sperimentale *109* 471 484 1959

3 *Davenport F M & Hennessy A V* The clinical epidemiology of Asian influenza Ann Int Med *49* 493 501 1958

4 *Davoli R & Bartolomei Corsi O* Anticorpi verso virus dell influenza A Asia nella popolazione normale Sperimentale *107* 358 362 1957

5 *Harboe A* The haemagglutination inhibiting antibodies in ferrets crossinfected with influenza A_2 virus and strains of the other A types Acta path et microbiol scandinav *49* 224 236 1960

6 *Harboe A* The heterologous anamnestic HI antibody increase resulting from cross infection *with influenza A and Dutch 56 A_1 strains* Acta path et microbiol scandinav 55 415–424 1962

7 *Harboe A* An anamnestic antibody increase in ferrets cross infected with influenza A_2 virus after myxoviruses not belonging to the A group Acta path et microbiol scandinav 55 425 430 1962

Curve B shows the mean reaction of 4 animals sensitized with tuberculin C It is an example of a moderate positive acute reaction, which disappears completely within 24 hours

Curve C shows the mean reaction of 4 animals sensitized with tuberculin C + TB wax It is an example of a more accentuated, acute response The reacting skin area shows transient cyanosis After a maximum at 30–45 minutes the infiltration decreases slowly, disappearing after 48–60 hours This means that at 36 and 48 hours a slight infiltration is still found Without a recording of the reaction in the interval between 60 minutes and 36 hours it might be difficult to decide *the nature of this infiltration As the reaction in the interval in question* however, shows a gradual decline, the probable explanation is, that the skin after a rather strong acute reaction needs more time to regain normal status The 36–48 hour infiltration seems to be a secondary effect of the acute reaction a slight *Arthus* phenomenon, and does not represent a delayed response This supposition is confirmed by a comparison with curve D

Curve D shows the mean reaction of 8 animals sensitized with heat killed tubercle bacteria It is an example of a slight acute reaction followed by a rather strong delayed reaction The first part of the curve looks like curve A a moderate acute reaction is seen with a maximum at 30 minutes, followed by a gradual decrease But at 6–12 hours the infiltration grows considerably, reaching another maximum at 24–48 hours After this the curve declines a slight infiltration still being found at 156 hours Two maximum periods of the reaction are clearly seen They are separated by an interval with only slight infiltration at 2–5 hours If delayed hypersensitivity was not present, a gradual return of the skin to the normal state would be expected at this time The new infiltration, which arises after 6–12 hours, signifies D hypersensitivity

These examples show the value of the intracutaneous reaction as a measure of D hypersensitivity, and especially as a method making possible a separation between delayed reactions and protracted acute reactions The basis of using the method for this separation is a frequent recording of the reaction during the whole response period

The acute cutaneous reactions give much interesting information about the I hypersensitivity induced by the various antigen mixtures It appears that in some cases the ileum contraction technique and the acute cutaneous reaction give divergent results This, however, is no matter of surprise The acute cutaneous reaction is a transient, intra vital process in a small area of tissue, which is continuously influenced by the reacting organism The ileum contraction experiment, on the contrary is a recording of the anaphylactic response of an isolated organ By applying the latter method the influence of several unknown circumstances is eliminated as the variable factors and individual differences in the interaction between the organism and the reacting skin area are out of the question It appears from the cutaneous control

ESTIMATION OF HYPERSENSITIVITY

The *I hypersensitivity* was estimated on the basis of an ileum contraction technique The reactivity was titrated with 1 0, 0 1, 0 01, 0 001 etc mg of antigen, and the reactions were registrated directly on a rotating cylinder The intensity of the I hypersensitivity, *s*, was expressed in the following way

$$s = -\log \frac{10}{t}$$

t being the smallest active dose of antigen The ileum of animals sensitized with products of tubercle bacteria was tested with tuberculin C

Furthermore the I hypersensitivity was studied on the basis of the acute, intracutaneous reactions

The *D hypersensitivity* was estimated on the basis of the delayed response to intracutaneous antigen injection

The *intracutaneous reactions* were carried out in the following way Animals sensitized with products of tubercle bacteria were all tested with three sorts of tuberculin· (1) a heated, $(NH_4)_2SO_4$-precipitated tuberculin (81), (2) an unheated concentrate of Sauton's medium, *tuberculin C, (described above), and* (3) a heated CCl_3COOH precipitated tuberculin (48) The dose of antigen was always 0 01 mg The cutaneous reactions were studied after 1, 2, 5, 10, 20, 30, 45, and 60 minutes and after 2, 6, 12, 24, 36, 48, 60, 72, 84, 108, and 156 hours

The intensity of the acute and delayed responses are in Table 1 indicated as +, + +, + + + etc This rather crude graduation is made on the basis of more elaborate, diagrammatic registrations of each animal In Fig 1 some typical examples of cutaneous reactions are seen

Curve A is a control showing the mean reaction of 10 normal animals It is expressive of the pure traumatic reaction to the intracutaneous antigen injection

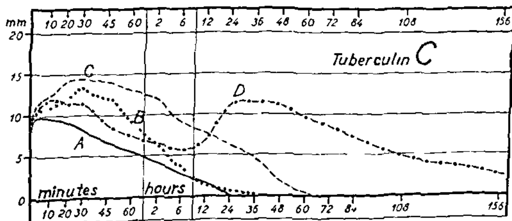


Fig 1

Intracutaneous Reactions of Guinea Pigs Sensitized with Injections of Tuberculin C (B), Tuberculin C + TB Wax (C) and Heat Killed Tubercle Bacteria (D) (A) Is a Control

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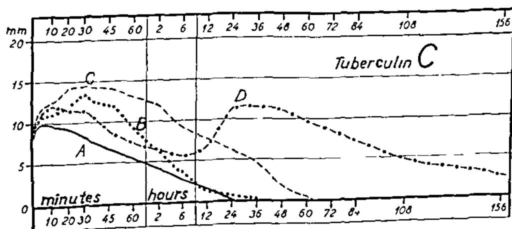


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Curve D shows the mean reaction of 8 animals sensitized with heat killed tubercle bacteria. It is an example of a slight acute reaction followed by a rather strong delayed reaction. The first part of the curve looks like curve A: a moderate acute reaction is seen with a maximum at 30 minutes, followed by a gradual decrease. But at 6–12 hours the infiltration grows considerably, reaching another maximum at 24–48 hours. After this the curve declines, a slight infiltration still being found at 156 hours. Two maximum periods of the reaction are clearly seen. They are separated by an interval with only slight infiltration at 2–5 hours. If delayed hypersensitivity was not present, a gradual return of the skin to the normal state would be expected at this time. The new infiltration, which arises after 6–12 hours, signifies D hypersensitivity.

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TABLE 1

No. of animals	Antigen mixture (dose per animal)	Liquid medium	mode of applic.	I type (cut.)	I type (ileum)	D type
16	heat killed TB (10 mg × 2)	NaCl 0.9%	inject	+	0.25	+++
12	heat killed TB (2 mg × 2)	paraff. oil	inject	++	3.50	+++++
12	delipidated TB (10 mg × 2)	NaCl 0.9%	inject	++	1.25	+
8	delipidated TB (2 mg × 2)	paraff. oil	inject	++	1.00	+++
16	delipidated TB + TB wax ((10 mg+10 mg) × 2)	NaCl 0.9%	inject	++	2.25	+++
8	delipidated TB + TB phosphatide ((10 mg+10 mg) × 2)	NaCl 0.9%	inject	++	1.25	(+)
8	tuberculin C (5 mg × 2)	NaCl 0.9%	inject	+	2.00	0
8	tuberculin C (5 mg × 2)	paraff. oil	inject	+++	1.25	0
8	tuberculin C + TB wax ((5 mg+10 mg) × 2)	NaCl 0.9%	inject	+++	1.75	0
8	tuberculin C + heat killed TB ((5 mg+10 mg) × 2)	NaCl 0.9%	inject	+	1.25	+++
8	delipidated TB + TB wax ((10 mg+10 mg) × 2)		implant	+	1.25	0
8	delipidated TB + TB phosphatide ((10 mg+10 mg) × 2)	–	implant	+	1.00	0
8	tuberculin C + TB wax ((5 mg+10 mg) × 2)		implant	+	2.00	0
8	tuberculin C + TB phosphatide ((5 mg+10 mg) × 2)	–	implant	+	1.66	0
8	TB wax (10 mg × 2)		implant	0	0	0
8	egg albumin (1 mg × 2)	NaCl 0.9%	inject	+++	2.75	0
8	egg albumin + TB wax ((1 mg+10 mg) × 2)	NaCl 0.9%	inject	+++++	4.00	0
8	egg albumin + heat killed TB ((1 mg+10 mg) × 2)	NaCl 0.9%	inject	with egg albumin +++ with tuberculin C +	 5.00 0.50	 0 +++
5	egg albumin + TB wax (1 mg+10 mg)		implant	++	6.00	0

reaction (curve A) that a considerable blind value is involved in the acute intracutaneous response In this respect the ileum contraction experiment is superior, as the ileum from a normal guinea pig does not react to antigen at all Furthermore, as the ileum reaction is recorded directly by the reacting tissue itself, there is no possibility of subjective measuring error For the reasons named above the estimation of I hypersensitivity in the following discussion and conclusions is mainly based upon the results of the ileum contraction experiments

Preliminary experiments showed that the small antigen doses used for the intracutaneous tests might give rise to I hypersensitivity manifesting itself as a positive ileum reaction Consequently animals on which an intracutaneous test had been performed were not used for ileum contraction experiments

In Table 1 the number of animals used for each experiment, and the dose and combination of the antigens or antigen mixtures are seen, and the mean values of the I and D hypersensitivity induced are given

On the basis of the presented, experimental evidence it is possible to put forward the following considerations

Whole, heat killed tubercle bacteria in 0 9 per cent NaCl induce D hypersensitivity, but no appreciable I hypersensitivity Paraffin oil markedly enhances the ability of tubercle bacteria to induce D hypersensitivity, and also enables them to induce I hypersensitivity

Delipidated tubercle bacteria The ability of tubercle bacteria to induce D hypersensitivity is considerably reduced, when the lipids are extracted, and at the same time the bacteria acquire the ability to induce I hypersensitivity The ability of delipidated tubercle bacteria to produce hypersensitivity is enhanced by the wax fraction, and this effect applies to the D type as well as to the I type The phosphatide fraction does not affect the antigenic effect The ability to induce I hypersensitivity is not enhanced by paraffine oil, the D type is somewhat intensified

[illegible] with

p [illegible] ivity

T [illegible] lipid solvents including paraff[illegible]

[illegible] the enhancing effect of paraffin oil on I hypersensitivity actually depends on a paraffin oil soluble lipid fraction, which has been removed from the tubercle bacteria at the delipidation, and is probably a part of the wax fraction The wax fraction equally intensifies I and D hypersensitivity, and the experiments do not indicate any special connection between the wax fraction and the development of D hypersensitivity

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8	tuberculin C + TB wax ((5 mg+10 mg) × 2)		implant	+	2.00	0
8	tuberculin C + TB phosphatide ((5 mg+10 mg) × 2)		implant	+	1.66	0
8	TB wax (10 mg × 2)		implant	0	0	0
8	egg albumin (1 mg × 2)	NaCl 0.9%	inject	+++	2.75	0
8	egg albumin + TB wax ((1 mg+10 mg) × 2)	NaCl 0.9%	inject	+++++	4.00	0
8	egg albumin + heat killed TB ((1 mg+10 mg) × 2)	NaCl 0.9%	inject	with egg albumin +++	5.00	0
				with tuberculin C +	0.50	+++
5	egg albumin + TB wax (1 mg+10 mg)		implant	++	6.00	0

DISCUSSION

It appears that the wax fraction of tubercle bacteria has an ability to intensify the I hypersensitivity as well as the D hypersensitivity. The wax fraction seems merely to *enhance* the existing ability to induce I or D hypersensitivity. In these experiments there is no evidence that the wax fraction could convey to any antigenic mixture the actual ability to induce D hypersensitivity. The activity of the wax fraction affects both types of hypersensitivity and is presumably an enhancing influence on the antigenic effect itself. The effect of the antigen thus enhanced subsequently leads to a hypersensitivity of the I type or the D type, depending on other factors.

As this seems to indicate that the hypersensitivity following an antigenic activity is intensified by the wax fraction, it is remarkable that whole tubercle bacteria do not, in spite of their wax content, induce I hypersensitivity. A comparison between the antigenic effect of whole, delipidated, and paraffin oil-suspended tubercle bacteria gives rise to the suggestion that the lipids function as a "barrier" between the soluble antigens of the tubercle bacterium and its surroundings. This "barrier" prevents whole tubercle bacteria from inducing I hypersensitivity in spite of the enhancing influence of the wax fraction. It would seem reasonable to associate this effect with the ability to induce D hypersensitivity also.

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It appears that paraffin oil enhances the hypersensitivity only in cases where the antigen mixtures contain tuberculolipids. Paraffin oil can dissolve the wax component, "Pmko", which is identical with wax D (3, 13, 14, 15, 16). In a paraffin oil suspension of whole tubercle bacteria, the oil phase will therefore contain this active component, which, being dissolved in a large, neutral medium, is subsequently dispersed in the organism in the way characteristic of paraffin oil (75, 80). The wax fraction, which is thus widely distributed and steadily deposited, will cause a strong enhancement of the antigenic effect of the tubercle bacteria contained in the oil; this applies to the I type as well as the D type (17, 18, 24, 29, 31, 47, 57, 60, 61, 78, 79). If the oil has also emulsified water drops in it, containing antigen, this antigen will likewise acquire a markedly enhanced activity. The effect of *Freund*'s "adjuvant" (27, 29, 30, 31, 32, 33) can probably be explained in this way. The paraffin oil does not exert any influence in itself, but merely acts as a solvent for the tubercle bacterial wax [illegible]

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Implantation of various antigen combinations Some experimental evidence may suggest that the lipids of the tubercle bacterium function as a "barrier" between the soluble antigens of the bacterium and its surroundings This appears *inter alia* from a comparison between the ability of whole and delipidated tubercle bacteria to induce I hypersensitivity The contact between antigen and tissues may be modified by this "barrier", which thus may be of importance to the rise of D hypersensitivity To investigate this possibility antigen combinations were formed in small pellets, which were implanted subcutaneously in guinea pigs A special antigenic situation, characterized by a slow passage of the antigen to tissue constantly influenced by the lipid fraction, was probably brought about in this way

The implantation experiments show that the slight ability of delipidated tubercle bacteria to induce D hypersensitivity fades away, if the bacteria are combined with the wax fraction in an implantate It is interesting to observe, that the ability to induce I hypersensitivity is retained under these conditions

Implantates consisting of tuberculin + wax or tuberculin + phosphatide do not induce D hypersensitivity, but considering the poor resorption of the implantates from the subcutaneous tissue, a rather strong I hypersensitivity may be said to be induced

Implantation of the wax fraction does not induce any demonstrable hypersensitivity

The protracted, simultaneous contact of tissue, lipid and antigen appears to be of no importance to the aetiology of D hypersensitivity The implantation experiments do not indicate any connection between the wax fraction and the development of D hypersensitivity

Egg albumin induces a strong I hypersensitivity, which is markedly enhanced by the wax fraction and by whole tubercle bacteria These antigen mixtures, however, do not induce D hypersensitivity to egg albumin, neither when given as injection nor as implantate

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results are in good agreement with other studies of this topic As many small, unrecorded, technical differences may occur in these rather complicated experiments, an exhaustive discussion of the interpretation of the divergent results will be hardly worth while

In this study a special point is made of the concurrent recordings of the I type as well as the D type of hypersensitivity The overall result of the examinations and considerations indicates that the activity of the wax fraction can hardly be to convey to an accompanying antigen *the ability to induce D hypersensitivity* As far as can be seen, the wax fraction is of no importance for the *type* of hypersensitivity, it seems merely to intensify the hypersensitivity induced by the antigenic potency already present This intensification applies to both types of hypersensitivity, the I type as well as the D type

SUMMARY

Antigen mixtures of varied composition, containing whole tubercle bacteria, delipidated tubercle bacteria, "purified wax" or "crude phosphatide" of tubercle bacteria, tuberculin, egg albumin, or paraffin oil were injected or implanted subcutaneously in guinea-pigs The purpose was to gain some experimental evidence concerning the I and D types of hypersensitivity induced by antigen mixtures containing products of tubercle bacteria A special point was made to assess the activity of the wax fraction as regards the development of D hypersensitivity Consequently concurrent recordings of the I type and the D type were carried out

The results of the examinations and considerations are indicated below

1) The wax fraction seems to be of no importance for the *type* of hypersensitivity

2) The wax fraction seems merely to *enhance* the existing ability to induce I or D hypersensitivity

3) Lipid solvents, including paraffin oil, cause an alteration in tubercle bacteria, which renders them capable of inducing I hypersensitivity

4) At delipidation with neutral, organic solvents the ability of tubercle bacteria to induce D hypersensitivity decreases, but is preserved

5) In water-in-paraffin oil emulsions containing tubercle bacteria in the oil phase (*Freund*'s "adjuvant"), the paraffin oil does not exert any influence in itself but merely acts as a solvent for the tubercle bacterial wax fraction, which possesses the actual ability to enhance hypersensitivity

6) It seems probable that the ability to induce D hypersensitivity is primarily connected with some quality in the bacterial body itself, and not with any single fraction

REFERENCES

1 *Anderson R J* The Separation of the Lipoid Fractions from Tubercle Bacilli J Biol Chem 74 525 1927
2 *Anderson R J* A Study of the Phosphatide Fraction of Tubercle Bacilli J Biol Chem 74 537 1927
3 [illegible]
4 [illegible]
5 *Billingham R E Brent L & Medawar P B* Actively Acquired Tolerance of Foreign Cells Nature London 172 603 1953
6 *Bindslev G* Overfølsomhed og Immunitet ved Tuberkulose Dissertation Busck Copenhagen 1939
7 *Bohrod M C* Pathology of Allergic and Collagen Diseases Prigal S J (ed) Fundamentals of Modern Allergy McGraw Hill Book Comp New York 1960 p 107
8 [illegible]
9 [illegible]
10 [illegible]
Symposium London 1959 p 555
11 *Burnet M* Autoimmune Disease M J Australia 49 1 1962
12 *Chase M W* Models for Hypersensitivity Studies Lawrence H S (ed) Cellular an Humoral Aspects of the Hypersensitive States Symposium New York 1959 p 251
13 *Choucroun N* Sur un antigene sensibilisant extrait du bacille tuberculeux C.R. Acad Sci Paris 208* 1757 1939
14 *Choucroun N* Biological Effects of a Toxic and a Sensitizing Substance Isolated from Paraffin Oil Extract of Dead Tubercle Bacilli Science 98 327 1943
15 *Choucroun N* Biological Properties of two Substances Isolated from Paraffin Oil Extract of Dead Tubercle Bacilli Am Rev Tuberc 56 203 1947
16 *Choucroun N* Antigenic Components of the Tubercle Bacillus in Relation to Tuberculin Hypersensitivity and Immunity Internat Arch Allergy 9 291 1956
17 *Corper H J & Cohn M L* The Effect of Paraffin Hydrocarbons on Tuberculoallergy and Tuberculoimmunity Produced by Tubercle Bacilli Am J Clin Path 12 73 1942
18 *Coulaud F* Allergie realisée avec des bacilles tuberculeux et morveux morts enrobés dans le paraffine Pouvoir vaccinant des bacilles de Koch ainsi préparés Bull Acad nat méd 115 232 1936
19 *Crowle A J* A Study of the Immunizing Factors of the Tubercle Bacillus Dissertation Stanford Univ 1954 Abst Dissertation Abstracts 14 1499 1954
20 *Crowle A J* Immunizing Constituents of the Tubercle Bacillus Bact Rev 22 183 1958
21 *Dienes L* The Immunological Significance of the Tuberculous Tissue J Immun \ 15 141 1928
22 *Dienes L* Further Observations Concerning the Sensitization of Tuberculous Guinea Pig [illegible]
23 *Dienes L* [illegible]
24 *Dienes L* [illegible] tul er [illegible] dans [illegible]
25 *Eisen H N* [illegible] C A [illegible] Lon [illegible]
26 *Eisen H N* Hypersensitivity to simple Chemicals Lawrence H S (ed) Cellular and Humoral Aspects of the Hypersensitive States Symposium New York 1959 p 89

27 *Fischel E F Kabat E A Stoerk H C & Bezer A E* The Role of Tubercle Bacilli in Adjuvant Emulsions on Antibody Production to Egg Albumin J Immunol 69 611 1952

28 *Forney J E* The Sensitization of Guinea Pigs to micrococcus pyogenes var aureus in the Presence of the "Wax" of Acid fast Bacilli Am Rev Tuberc 69 241 1954

29 *Freund J* Some Aspects of Active Immunization Am Rev Microbiol *1* 291 1947

30 *Freund J* The Mode of Action of Immunologic Adjuvants Advances Tuberc Res 7 130 1956

31 *Freund J Casals J & Hosmer E P* Sensitization and Antibody Formation after Injection of Tubercle Bacilli in Paraffin Oil Proc Soc Exper Biol & Med 37 509 1937

32 *Freund J & Stone S H* Unpublished Experiment Lawrence H S (ed) Cellular and Humoral Aspects of the Hypersensitive States Symposium New York 1959 p 243

33 *Freund J Thomson K J Hough H B Sommer H E & Pisani T H* Antibody Formation and Sensitization with the Aid of Adjuvants J Immunol *60* 383 1948

34 *Friis T* Tuberkulinfølsomheden hos Marsvin Dissertation Gad Copenhagen 1953 p 114

35 *Hansen F* Absolute und relative zellulare und humorale Immunität bei bakteriellen Infektionen einschliesslich der Tuberkulose Med Welt *11* 209 1961

36 *Harthausen H* The Pathogenesis of Allergic Eczema Elucidated by Transplantation Experiments on Identical Twins Acta dermat venereol 23 438 1943

37 *Harthausen H* The Occurrence of Humoral Antibodies in Allergic Eczema Investigated through Parabiosis Experiments on Guinea Pigs Acta dermat venereol *24* 286 1944

38 *Harthausen H* The Pathogenesis of Allergic Eczema Illustrated by Transplantation Experiments Acta dermat venereol *31* 1 1951

39 *Harthausen H* Attempts on Passive Local Sensitization by Intracutaneous Injection of Cells from Freshly Excised Lymph Nodes of Eczema Allergics J Invest Dermat *21* 237 1953

40 *Jensen K A* Relation entre l'allergie et l'immunité dans la tuberculose VII Conférence de l'Union internationale contre la tuberculose 1932 p 71

41 *Jensen K A* Essai de standardisation des méthodes de laboratoire Deuxième rapport de la sous commission des méthodes de laboratoire Bull Un int Tuberc 25 96 1955

42 *Jensen K A* Infektionspatologi F a d L Copenhagen 1959

43 *Jensen K A & Bindslev G* Experimental Studies on the Development of Tuberculous Infection in Allergic and Non Allergic Animals Acta tuberc scandinav *11* 101 1937

44 *Jensen K A Bindslev G & Holm J* Experimental Studies on the Development of Tuberculous Infection in Allergic and Non Allergic Animals Acta tuberc scandinav *9* 27 1935

45 *Jensen K A Bindslev G Møller S Hansen A & Lind P* Old Tuberculin and Purified Tuberculin Tubercle *19* 386 1938

46 *Kabat E A Mayer M M & Heidelberger M* Experimental Immunochemistry Springfield 1948 p 448

47 *Kallós P* Weitere Beiträge über die biologischen Eigenschaften des Tuberkulins Untersuchungen über den anaphylaktischen Shock bei tuberkulösen Tieren und über die Tuberkulin Allgemeinreaktion Acta med scandinav *109* 115 1941

48 *Landsteiner K & Chase M W* Skin Sensitization by Intraperitoneal Injections J Exper Med *71* 237 1940

49 *Larson C L Ribi E Wicht W C & List R* Skin Reactions Produced in Rabbits by Cell Walls and Protoplasm of mycobacterium tuberculosis and mycobacterium butyricum Am Rev Resp dis 83 184 1961

50 *Lawrence H S* Delayed Bacterial Allergy Homograft Sensitivity and Autoimmune Disease New York J Med 62 356 1962

51 *MacLeod C M* Hypersensitivity and Disease Lawrence H S (ed) Cellular and Humoral Aspects of the Hypersensitive States Symposium New York 1959 p 615

52 *Medawar P B* Transplantation Immunity and Subcellular Particles Ann New York Acad Sc 1 68 1957

53 *Medawar P B* The Immunity of Transplantation The Harvey Lectures New York 52 144 1956 57

54

55

56 *Myrvik Q* & *Weiser R S* The Tuberculin Reaction V The Antigenicity of Chloroform Soluble Tuberculoprotein Wax J Immunol 68 413 1952

57 *Møller P* Experimentelle Undersøgelser over Tuberculoseimmunitet Dissertation Munksgaard Copenhagen 1947

58 ensitivity to ... al Aspects of

59 ... njection with ... Mechan

60 ... Arch

61 ... with ... Observations on the Frequency of Anaphylactic Sensitization during

62 Poun

63 *Raffel S* The Relationship of Acquired Resistance Allergy Antibodies and Tissue Reactivities to the Components of the Tubercle Bacillus Am Rev Tuberc 54 564 1946

64 *Raffel S* The Components of the Tubercle Bacillus Responsible for the Delayed Type of "Infectious" Allergy J Infect Dis 82 267 1948

65 Raff

66

67

68

69 ... to Tuberculosis ... Foundation Symposium on Experimental Tuberculosis London 1955 p 261

70 *Raffel S Arnaud L E Dukes C D & Huang J S* The ...

71 *Raff*

72 *Raffel S & Forney J E* Hypersensitivity to a Simple Ch ...

73

74

75

76 ... bacilles tuberculeux morts ... Res 5 91 1951

77 Sabin ... Studies on Tuberculosis J Exper Med 52 suppl 3 1 1930

78 *Saentz A* Accroissement de l'état allergique et titrage de la sensibilité tuberculinique conférés au cobaye par l'inoculation sous cutanée de bacilles tuberculeux enrobés dans l'huile de vaseline C R Soc Biol Paris *120* 1050 1935

79 *Saentz A* Sur le méchanisme de l'augmentation de l'allergie produits par l'enrobage des bacilles morts dans l'huile de vaseline C R Soc Biol Paris *125* 714 1937

80 *Saentz A & Canetti G* Lésions et troubles morbides singuliers engendrés par les bacilles tuberculeux morts enrobés dans l'huile de vaseline Presse méd *47*1 849 1939

81 *Seibert F B & Glenn J T* Tuberculin Purified Protein Derivative Am Rev Tuberc *44* 9 1941

82 *Smith D W & Kubica G P* Comparison of Extracts of Tubercle Bacilli and BCG as Immunizing Agents in Guinea Pigs Proc Soc Exper Biol & Med *90* 629 1955

83 *Stetson C A* The Role of Antibody in the Rejection of Homografts Schaeffer J H LoGrippo G A & Chase M W (eds) Mechanisms of Hypersensitivity Symposium London 1959 p 569

84 *Suter F & White R G* The Response of the Reticuloendothelial System to the Injection of the Purified Wax and the Lipopolysaccharide of Tubercle Bacilli Am Rev Tuberc *70* 793 1954

85 *Toda T Kato M Hashimoto T & Tsuru M* Protective Inoculation Experiments with the Phosphatides and Wax Substances Extracted from Tubercle Bacilli II Immunological Studies of the Various Constituents of the Tubercle Bacillus Bodies Jap Lit Tuberc Forsch 4 43 1934 Chem Abstr *30* nr 8359 1936

86 *U S Public Health Service* Experimental Studies of Vaccination Allergy and Immunity in Tuberculosis VI Comparative Effects of BCG H37Ra myco bacterium phlei and a Wax protein Fraction of Human Tubercle Bacilli Am J Hyg *62* 270 1955

87 *Waksman B H* Experimental Allergic Encephalomyelitis as Prototype of the Class of Autoallergic Diseases Schaeffer J H LoGrippo G A & Chase M W (eds) Mechanisms of Hypersensitivity Symposium London 1959 p 679

88 *Waksman B H & Adams R D* Tubercle Bacillus Lipopolysaccharide as Adjuvant in the Production of Experimental Allergic Encephalomyelitis in Rabbits J Infect Dis *93* 21 1953

89 *Weiss D W* Inhibition of Tuberculin Skin Hypersensitivity in Guinea Pigs by Injection of Tuberculin and Intact Tubercle Bacilli during Fetal Life J Exper Med *108* 83 1958

90 *White R G* The Adjuvant Effect of Mycobacterial Cells and Fractions Schaeffer J H LoGrippo G A & Chase M W (eds) Mechanisms of Hypersensitivity Symposium London 1959 p 637

91 *White R G Bernstock L Johns R G S & Lederer F* The Influence of Components of m tuberculosis and other Mycobacteria upon Antibody Production to Ovalbumin Immunology *1* 54 1958

92 *White R G Coons A H & Connolly J M* Studies on the Antibody Production IV The Role of the Wax Fraction of mycobacterium tuberculosis in Adjuvant Emulsions on the Production of Antibody to Egg Albumin J Exper Med *102* 83 1955

93 *White R G & Marshall A H E* The Role of Various Chemical Fractions of m tuberculosis and other Mycobacteria in the Production of Allergic Encephalomyelitis Immun 1 gy 2 111 1958

94 *Zinsser H* Studies on the Tuberculin Reaction and on Specific Hypersensitiveness in Bacterial Infection J Exper Med 34 495 1921

95 *Zinsser H & Tamiya T* An experimental Analysis of Bacterial Allergy J Exper Med 44 753 1926

COMPARATIVE INVESTIGATIONS OF THE SENSITIVITY OF N GONORRHOEAE TO PENICILLIN

By

ALICE REYN MICHAEL WEIS BENTZON and HANS ERICSSON

Received 18 x 62

Many investigators have observed a deterioration in the results of the treatment of gonorrhoea with the penicillin doses hitherto employed (2 3 11 12 13 14 15 18 20) and several laboratories (16 21 22 25 27) have reported on strains of gonococcus less sensitive *in vitro* to antibiotics and especially to penicillin In addition several workers have demonstrated a positive correlation between failures of treatment and *in vitro* results In 1958 it was observed by *Reyn et al* that the number of less sensitive strains was significantly greater among strains sent in with a request for sensitivity determination than among those received without such a request

During recent years the incidence of gonorrhoea has increased in many countries (4 10 13 15 18 19) and the increase has been most marked among female teenagers (26 29) The complication rate is still of a considerable size (10) In order to counteract the difficulties described above on 10th June 1960 a committee on the culture and sensitivity determination of gonococci was established the initiative for this being taken by the Royal Swedish Health Service Within a short time the committee was supplemented so that it comprised eight members[1] from four Scandinavian countries Denmark Finland Norway and Sweden In the following the results of comparative experiments performed in six laboratories (designated as labs 1 6) are described The purpose of the experiments was amongst other things to agree upon a reference method for determination of sensitivity and also to work out a method suitable for routine examinations

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The reference method was intended for use in checking the local methods and also for the "translation" of results of a diffusion method, measured in mm diameter of inhibition zone to concentrations By definition, a dilution method should be chosen as the reference standard to which the other methods may be related. Furthermore, a method suitable for current epidemiological investigations was desirable for surveillance of the situation with regard to the sensitivity of the gonococcal strains in circulation.

TABLE 1

Results of Preliminary Tests Using the Plate Dilution Method
IC_{50} = 50 per Cent Inhibitory Concentrations in µg per ml

Strain nos	Orig nos	Isolated in	Penicillin*	Streptomycin	Sulphathiazole	Tetracycline	Chloramphenicol
Dr. Thayer's strains							
Gc I	P-1	1958	0 21	≤ 1 41	4 2	0 37	0 39
Gc II	*P-5*	1958	0 053	1 68	3 6	0 37	≤ 0 28
Gc III	P-26	1955	0 026	*1 19*	*5 0*	*0 22*	≤ *0 28*
Gc IV	P-78	1960	0 71	> 2000	2 1	0 88	0 46
Gc V	*P-83*	*1960*	0 71	> 2000	6 0	0 88	0 39
Dr Reyn's strains							
Gc VI	11413	1940	0 0047	≤ 1 41	≤ 0 75	0 31	0 33
Gc VII	11421	1940	0 0054	*2 8*	≤ *0 75*	*0 22*	≤ *0 28*
Gc VIII	12990	1960	≥ 0 43	4 0	34 0	≥ 1 77	1 31
Gc IX	*17732*	*1940*	*0 0066*	*3 4*	≤ *0 75*	*0 26*	≤ *0 28*
Gc X	41826	1960	0 0066	2 4	3 0	0 22	≤ 0 28
XI Staph	FDA 209 P		0 0079	1 68	*48 0*	*0 22*	*2 2*
XII Sarc	Lut ATCC 9341		0 0042	4 0	3 0	0 53	0 78

Strains I-V tested in lab 1 14/6 60 with penicillin streptomycin, sulphathiazole and tetracycline All str received from Dr J D Thayer, V D R L, Chapel Hill, N Carolina U S A
Strains VI-XII tested in lab 1 31/1 61 with streptomycin sulphathiazole and tetracycline
Strains VI-XII tested in lab 1 26/8 60 with penicillin (except str XI tested 12/1 61)
Strains I XII tested in lab 1 21/4 61 with chloramphenicol

* 1 IU = 0 6 µg

MATERIAL AND METHODS

Experimental cultures a) 10 gonococcal strains 5 of which were isolated in the *Neisseria department of Statens Seruminstitut* Copenhagen and 5 by *Dr James, D Thayer*[1], Chapel Hill, U S A Previously all the strains had been examined by both *Thayer and Reyn* who had each used both their own technique and that of the other[2] b) Staphylococcus aureus (FDA 209 P) c) Sarcina lutea (ATCC 9341) Cultures were *lyophilized in one of the laboratories and two ampoules* of each culture were sent to the other laboratories By means of the plate dilution method naturally occurring strains of gonococcus were so chosen that the variation in their sensitivity to penicillin was as great as possible

1 Present address V D R L, USPHS, Communicable Disease Center Atlanta 22 Georgia, U S A
2 To be published (24).

in another paper

Plate dilution method The technique was that previously described in brief by *Reyn, Korner & Bentzon* (21), it is at present routinely used in the Neisseria department of Statens Seruminstitut, Copenhagen *Media* In the present study both a common "standard medium" (S) and a local "routine medium" (R) were employed Table 2 shows the compositions of the two media It is seen that R differs only slightly from S, which contained 1) about 83 per cent *placental broth* (produced in the local laboratories according to a common recipe and with the same batch of bacto agar (Difco) and of peptone (Orthana Special), 2) about 7 per cent defibrinated or citrated horse blood, obtained locally, 3) 10 per cent sterile, pooled horse serum delivered by one of the laboratories and 4) varying amounts of penicillin G (Leo)

TABLE 2

Composition of Media

	Agar		Broth					Blood*	Ascitic fluid or serum
	Brand	%	Source	% NaCl	% Na_2HPO_4	Peptone %	Peptone Brand	%	%
Standard medium	Bacto	1 1	Human placenta	0.3	0 2	1 0	Orthana Special	6 67	10 0§
Routine media									
Lab 1	Japan	1 1	Ox heart	0 3	0 2	0 0		6 67	10 0†
Lab 2	Japan	1 3	Ox meat	0 3	0 2	1 0	Bacterial Peptone (Wilson)	8 5	7 1§
Lab 3	Japan	1 3	Human placenta	0 3	0.2	1 0	UCLAF	8 0	25 0†
Lab 4	Japan	1 2	Ox meat	0 3	0 2	1 0	Witte	6 67	33 0†
Lab 5	Bacto	0 8	Human placenta	0 3	0 2	1 0	Orthana	6 0	11 0†
Lab. 6	Davis New Zeal	0 9	Combined meat extracts	0 3	0 2	1 0		7 3	13 0†

Agar blood serum and ascitic fluid final percentages
NaCl Na_2HPO_4 and peptone percentages in broth
* Defibrinated or citrated horse or human blood
§ Horse serum
† Ascitic fluid

The reference method was intended for use in checking the local methods and also for the "translation" of results of a diffusion method, measured in mm diameter of inhibition zone to concentrations. By definition, a dilution method should be chosen as the reference standard to which the other methods may be related. Furthermore, a method suitable for current epidemiological investigations was desirable for surveillance of the situation with regard to the sensitivity of the gonococcal strains in circulation.

TABLE 1

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IC_{50} = 50 per Cent Inhibitory Concentrations in µg per ml

Strain nos	Orig nos	Isolated in	Penicillin*	Strepto mycin	Sulpha thiazole	Tetra cycline	Chloram phenicol
Dr Thayer's strains							
Gc I	P-1	1958	0.21	≤1.41	4.2	0.37	0.39
Gc II	P-5	1958	0.053	1.68	3.6	0.37	≤0.28
Gc III	P-26	1955	0.026	2.19	5.0	0.22	≤0.28
Gc IV	P-78	1960	0.71	>2000	2.1	0.88	0.46
Gc V	P-83	1960	0.71	>2000	6.0	0.88	0.39
Dr Reyn's strains							
Gc VI	11413	1940	0.0047	≤1.41	≤0.75	0.31	0.33
Gc VII	11421	1940	0.0054	2.8	<0.75	0.22	≤0.28
Gc VIII	12990	1960	≥0.43	4.0	34.0	≥1.77	1.31
Gc IX	17732	1940	0.0066	3.4	≤0.75	0.26	≤0.28
Gc X	41826	1960	0.0066	2.4	3.0	0.22	≤0.28
XI Staph	FDA 209 P		0.0079	1.68	48.0	0.22	2.2
XII Sarc lut	ATCC 9341		0.0042	4.0	3.0	0.53	0.78

Strains I–V tested in lab 1 14/6 60 with penicillin, streptomycin, sulphathiazole
and tetracycline. All str. received from
Dr J D Thayer, V D R L, Chapel Hill,
N Carolina, U S A.
Strains VI–XII tested in lab 1 31/1 61 with streptomycin, sulphathiazole and tetracycline.
Strains VI–XII tested in lab 1 26/8 60 with penicillin (except str. XI tested 12/1 61).
Strains I–XII tested in lab 1 21/4 61 with chloramphenicol.

* 1 IU = 0.6 µg

MATERIAL AND METHODS

Experimental cultures: a) 10 gonococcal strains, 5 of which were isolated in the *Neisseria department of Statens Seruminstitut*, Copenhagen, and 5 by Dr James D Thayer[1], Chapel Hill, U S A. Previously, all the strains had been examined by both *Thayer and Reyn who had each used both their own technique and that of the other*[2]. b) Staphylococcus aureus (FDA 209 P). c) Sarcina lutea (ATCC 9341). Cultures were lyophilized in one of the laboratories and two ampoules of each culture were sent to the other laboratories. By means of the plate dilution method naturally occurring strains of gonococcus were so chosen that the variation in *their sensitivity* to penicillin was as great as possible.

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pond to the dilution, which gives growth degree 2, the corresponding penicillin concentration would be IC_0. Thus, *dilutions instead of concentrations* are used, and this means that large figures mean high sensitivity by both methods, large zones of inhibition being seen in sensitive strains (N_{50} = 0, 1, 2 etc corresponds to IC_{50} = 1, 0 5, 0 25 IU per ml) It must be emphasized that 2 does not mean 50 per cent of the original number of colonies, this gradation should merely serve as a practical experimental method of reading

TABLE 3

Determination of IC_{50} by means of the Karber Method
Crystalline Penicillin G 0 002 to 2 IU 11 Plates

Plate nos	Sum*	IU	µg	Plate nos	Sum	IU	µg
1	0	≤0 0014	≤0 0008		23	0 074	0 045
	1	0 0016	0 0010		24	0 088	0 053
	2	0 0020§	0 0012	7	25	0 105	0 063
	3	0 0023	0 0014		26	0 125	0 075
	4	0 0028	0 0017		27	0 149	0 089
2	5	0 0033	0 0020		28	0 177	0 106
	6	0 0039	0 0023	8	29	0 21	0 126
	7	0 0046	0 0028		30	0 25	0 150
	8	0 0055	0 0033		31	0.30	0 178
3	9	0 0066	0 0039		32	0.35	0 21
	10	0 0078	0 0047	9	33	0 42	0 25
	11	0 0093	0 0056		34	0 50	0 30
	12	0 0111	0 0066		35	0 59	0.36
4	13	0 0131	0 0079		36	0 71	0 42
	14	0 0156	0 0094	10	37	0 84	0 50
	15	0 0186	0 0112		38	1 00	0 60
	16	0 022	0 0133		39	1 19	0 71
5	17	0 026	0 0158		40	1 41	0 85
	18	0 031	0 0188	11	41	1 68	1 0
	19	0 037	0 022		42	2 00	1.2
	20	0 044	0 027		43	2 4	1 4
6	21	0 053	0 032		44	2 8	1 7
	22	0 063	0 038				

* The sum is obtained by ad i ng the a [illegible]
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be alter [illegible]
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each IU [illegible]
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The degrees of gr [illegible]

Placental broth and preparation of plates Fresh, human placentae are freed from the umbilical cords and the coverings No antiseptics should be added when transported from the hospital to the laboratory The placentae are cut into pieces and rinsed in cold tap water After coarse chopping in a machine two litres of tap water per kg is added and the mixture is stored overnight at about 4° C Next day the soup is boiled for 15 minutes after which the placental tissue is separated from the broth The following ingredients are added 1 per cent Orthana peptone Special 0 3 per cent NaCl and 0 2 per cent Na_2HPO_4 12 H_2O The soup is boiled again made alkaline (pH 7 2), filtered and measured 15 g Bacto agar is added per liter, after which the broth is dispensed in flasks and autoclaved at 120° C for 20 minutes After autoclaving pH will be about 7 2 Store in a cool place The placental broth agar is melted in a waterbath at 80° C, 185 ml amounts are dispensed in small flasks and maintained at 80° C 15 ml horse blood is added and the mixture is stirred continuously until it becomes chocolate brown, and is about to curdle (5 to 6 minutes) Because of the small amounts used the heating time given here is shorter than usual The medium is cooled to about 56° C and 25 ml sterile horse serum preheated to 56° C is added Finally, the various doses of penicillin are added (See below)

Penicillin solvent Na_2HPO_4, 2 H_2O 4 g, NaH_2PO_4 2 H_2O 7 g, aqua destill ad 1 liter Sterilize by autoclaving for 20 minutes at 120° C 5 ml sterile solvent is added to an ampoule of penicillin G (Leo) containing 100 000 international units (IU), resulting in a solution with 20 000 IU per ml From this solutions diluted with the solvent to contain 100, 10, and 1 IU ($1 + 199 \rightarrow 1 + 9 \rightarrow 1 + 9$) are prepared and suitable amounts of these are added to the small flasks containing the medium so that final concentrations from between 0 002 and 2 IU per ml (in 2 fold steps) are obtained (0 0012 to 1 2 ug per ml) Immediately after the addition of penicillin 4 mm thick plates are poured If 225 ml medium is too much half portions may be used, but it is safer with an excess When stored in a refrigerator the plates can be kept for about a week but it is recommended that the experiments be performed as quickly as possible after preparing the plates Plates without antibiotics serve as controls and are used in the disc method

Inoculum The growth from 18–20 hours old plate cultures are suspended in broth to contain about 10^9 viable units per ml The suspension is diluted 1 + 4 in broth using a 2 mm wire loop about 0 005 ml of this suspension is spread on a 1 2 cm sector of a plate 6 strains are inoculated on each plate and in order to minimize the deleterious effect of suspension all inoculations with one strain are completed before the next one is suspended The plates are incubated for about 24 hours at 36° C in an 8 per cent CO_2 atmosphere

Readings of results By comparison with the growth on a control plate without antibiotic, the readings of the results are graded as follows

Confluent growth 4, confluent, but less dense than 4 3, granular growth ≥ 50 colonies 2, 1 to 50 colonies 1, no growth 0 By means of this gradation a symmetrical distribution of the degrees of gonococcal growth is obtained, degrees 1 and 3 occurring with equal frequency Consequently, degree 2 can be taken as the mid point With this presumption it is justifiable to use the Karber method[1] in calculating the mean inhibitory concentration When a log scale for the penicillin dilutions (referred to 1 IU) is employed, Karber values are obtained, which we have called here N_{50} *values* These values corres

[1] For details of the Karber method the reader is referred to *Finney D J* (1947) 'Probit Analysis p 39 University Press Cambridge In that publication percentages are dealt with for example survival percentages and the determination of 50 per [illegible] the method is employed [illegible]ntages 0 25 50 75 and [illegible]ctor is constant (in our [illegible] the sum of percentages

(sum of degrees) (Table 3)

pond to the dilution, which gives growth degree 2, the corresponding penicillin concentration would be IC_{50} Thus, *dilutions instead of concentrations* are used, and this means that large figures mean high sensitivity by both methods, large zones of inhibition being seen in sensitive strains (N_{50} = 0, 1, 2 etc corresponds to IC_{50} = 1, 0 5, 0 25 IU per ml) It must be emphasized that 2 does not mean 50 per cent of the original number of colonies this gradation should merely serve as a practical experimental method of reading

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	11	0 0093	0 0056		34	0 50	0 30
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	16	0 022	0 0133		39	1 19	0 71
5	17	0 026	0 0158		40	1 41	0 85
	18	0 031	0 0188	11	41	1 68	1 0
	19	0 037	0 022		42	2 00	1 2
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6	21	0 053	0 032		44	2 8	1 7
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* The sum is obtained by adding the degrees of growth for all plates

§ If dilution steps other than 2 fold steps are employed the table values should be altered accordingly If plates containing m
periment (e g 4 8 etc IU) the table
each time the sum is increased by 4 I
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should be divided by 4 and so on

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O_4, 12 H_2O The soup is boiled again, made alkaline
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1 For details of the Karber method the reader is referred to *Finney D J* (1947) 'Probit Analysis' p 39 University Press Cambridge In that publication percentages are dealt with for example survival percentages and the determination of 50 per cent values is based upon percentages In the present paper the method is employed on degrees of growth which have been translated to percentages 0 25, 50 75 and 100 The tabulation is especially simple when the dilution factor is constant (in our case = 2) the 50 per cent value then being dependent only on the sum of percentages (sum of degrees) (Table 3)

only those points from the average of all the laboratories have been joined to form the solid curve The results from the different laboratories are systematically located at somewhat different levels, those from lab 6 lying about one half dilution step lower, and those from lab 5 about one half dilution step higher than the average (Stippled curves) Both the differences in level and the individual deviations are small in relation to the total range of variation

TABLE 4

Comparison of the $\bar{N}_{50}$ Values Obtained in the 6 Laboratories (See page 4)
Plate Dilution Method Standard Medium

Strains	Laboratories 1	2	3	4	5	6	$\bar{N}_{50}$	
Gc I	1 83[a]	1 17	1 42	2 42	2 08	1 67	1 77[c]	
	0 06[b]	0 60	− 0 35	0 65	0 31	− 0 10		
Gc II	2 50	2 83	3 67	2 42	3 75	0 25	2 57	
	0 07	0 26	1 10	− 0 15	1 18	2 32		
Gc III	3 75	4 00	3 75	3 92	4 25	3 42	3 85	
	− 0 10	0 15	− 0 10	0 07	0 40	− 0 43		
Gc IV	0 42	0 13	0 50		0 42	0 25	0 29*	
	0 13	0 42	0 21		0 13	− 0 04		
Gc V	0 83	0 58	1 50		0 75	0 59	0 85*	range
	− 0 02	− 0 27	0 65		− 0 10	0 27		7 14
Gc VI	6 83	7 58	8 42	6 33	8 25	7 17	7 43	
	0 60	0 15	0 99	− 1 10	0 82	− 0 26		
Gc VII	6 75	7 00	7 00	6 08	7 67	6 42	6 82	
	0 07	0 18	0 18	− 0 74	0 85	− 0 40		
Gc VIII	0 92	0 25	0 17	2 08	0.92	− 0 25	0 68	
	0 24	0 43	− 0 51	1 40	0 24	− 0 93		
Gc IX	6 00	6 42	5 75	6 33	6 08	5 50	6 01	
	0 01	0 41	− 0 26	0.32	0 07	− 0 51		
Gc X	6 00	6 08	6 17	5 92	6 42	5 33	5 99	
	0 01	0 09	0 18	− 0 07	0 43	0 66		
Average	3 58	3 58	3 84	3 68*	4 06	3 03	3 63	
	− 0 04	− 0 05	− 21	0 05	0 43	0 59		

a Average of 3 N_{50} values See page 240
b Difference between 1 and 3
c Average of N_{50} values for all laboratories
* Calculated by the method of "missing plots"

The results obtained with *the disc method* are given in Tables 5 and 6 In Fig 2 the results using 20 IU penicillin are presented graphically (D = average from all laboratories) The total variation in sensitivity is about 20 mm for 20 IU and about 25 mm for 2 IU The sequence of the strains is the same as in Fig 1 and the course of the graph of the averages (solid curve) shows that the two methods ...

... of the results from lab 6 is lower and that from lab 5 is higher than that of the average

investigation it was sometimes necessary to correct the degrees The correction was performed using the following principles if, in the readings, more than one figure 1 (or more than one figure 3) occurred only the last figure 1 was calculated as 1 and the remainder as 0 (or the first figure 3 as 3 and the remainder as 4) The correction was performed in order to determine N_{50} as accurately as possible

The diffusion or disc method. The technique has previously been described by *Ericsson et al* (5, 6, 7, 8) The same bacterial suspensions and media were employed as in the dilution method However, in one of the laboratories (5) a lower inoculum was used in order to obtain "a dense but not confluent growth" The plates were "dried" for 20–30 minutes at 37° C, leaving no free moisture on their surfaces 1 5 ml suspension was poured and over each 10 cm plate the superfluous liquid was sucked up The filter paper discs contained exactly 2 and 20 IU penicillin (1 2 and 12 0 µg), 50 µg di-hydrostreptomycin, 50 µg tetracycline hydrochloride, 30 µg chloramphenicol and 2 4 mg sulphathiazole The number of discs per plate was adjusted in relation to the expected size of the inhibition zones After inoculation the discs were placed on the plates, which were left for 3 hours at room temperature before incubation (3 hours' prediffusion) After about 20 hours' incubation at 36–37° C in a CO_2-atmosphere the zone diameters were measured in mm 3 readings of each zone were taken, the average being used for the evaluation of the present experiments In the discussion, the method of reading will be dealt with in more detail

The structure of the experiment A total of 6 laboratories took part in the investigation Each laboratory was to examine the sensitivity of all 12 bacterial cultures to penicillin within a single day, using both methods and the S and, if possible, also the R (See under media) The tests were to be repeated on 3 different days and the whole procedure was to be duplicated including the preparation of fresh media, except the placental broth agar This scheme was followed in 3 laboratories (1, 3 and 5) In lab 2 the R was used for the disc method only In lab 4 the R and S were used on different days for both methods The R was employed for the dilution method on one day and the S on 2 days, in the disc method the R was used on 2 days and the S on 3 days Something similar applied to lab 6, where the R was used on 2 days and the S on 3 days for the disc and the dilution methods, respectively In addition, the tests were performed on different days by both methods

RESULTS

Table 4 shows the average results (3 days) for *the dilution method* obtained in the 6 laboratories using the S The averages ($\bar{N}_{50}$) from all laboratories for the individual strains vary between 0 29 and 7 43 or within a range of about 7 dilution steps In Fig 1 the strains are arranged on the abscissa in the order corresponding to their averages, and on the ordinate the corresponding $\bar{N}_{50}$ values are plotted The N_{50}-values from the individual laboratories are also plotted on the ordinate, but

only those points from the average of all the laboratories have been joined to form the solid curve The results from the different laboratories are systematically located at somewhat different levels those from lab 6 lying about one half dilution step lower, and those from lab 5 about one half dilution step higher than the average (Stippled curves) Both the differences in level and the individual deviations are small in relation to the total range of variation

TABLE 4

Comparison of the N_{50} Values Obtained in the 6 Laboratories (See page 5)
Plate Dilution Method Standard Medium

Strains	Laboratories 1	2	3	4	5	6	N_{50}	
Gc I	1 83[a]	1 17	1 42	2 42	2 08	1 67	1 77[c]	
	0 06[b]	0 60	0 35	0 65	0 31	0 10		
Gc II	2 50	2 83	3 67	2 42	3 75	0 25	2 57	
	0 07	0 26	1 10	0 15	1 18	2 32		
Gc III	3 45	4 00	3 75	3 92	4 25	3 42	3 85	
	0 10	0 15	−0 10	0 07	0 40	−0 43		
Gc IV	0 42	0 13	0 50		0 42	0 25	0 29*	
	0 13	0 42	0 21		0 13	0 04		
Gc V	0 83	0 58	1 50		0 75	0 58	0 85*	
	0 02	0 27	0 65		0 10	0 27		range
Gc VI	6 83	7.58	8 42	6 33	8 25	7 17	7 43	7 14
	0 60	0 15	0 99	1 10	0 82	0 26		
Gc VII	6 75	7 00	7 00	6 08	7 67	6 42	6 82	
	0 07	0 18	0 18	0 74	0 85	0 40		
Gc VIII	0 92	0 25	0 17	2 08	0 92	0 25	0 68	
	0 24	−0 43	0 51	1 40	0 24	0 93		
Gc IX	6 00	6 42	5 75	6 33	6 08	5 50	6 01	
	0 01	0 41	0 26	0 32	0 0[illegible]	0 51		
Gc X	6 00	6 08	6 17	5 92	6 42	5 33	5 99	
	0 01	0 09	0 18	0 07	0 43	0 66		
Average	3 58	3 58	3 84	3 68*	4 06	3 03	3 63	
	0 04	−0 05	.21	0 05	0 43	0 59		

a Average of 3 N_{50} values See page 240
b [illegible]
c

The results obtained with *the disc method* are given in Tables 5 and 6 In Fig 2 the results using 20 IU penicillin are presented graphically (D = average from all laboratories) The total variation in sensitivity is about 20 mm for 20 IU and about 25 mm for 2 IU The sequence of the strains is the same as in Fig 1 and the course of the graph of the averages (solid curve) shows that the two met[illegible]

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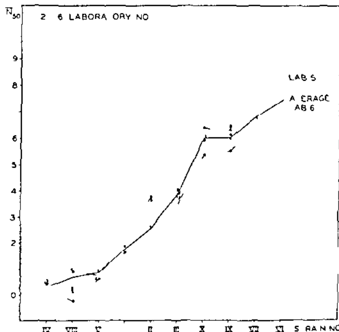

STANDARD MEDIUM
ATE D UT O METHOD
6 LABORA ORY NO
LAB S
A ERAGE
AB 6
S RA N NO

Fig 1

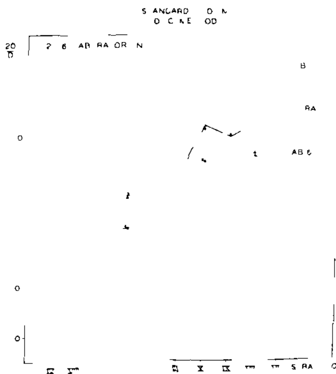

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Fig 2

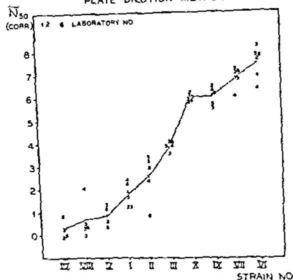


Fig 3

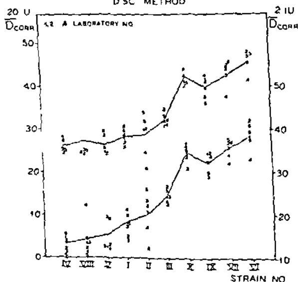


Fig 4

Fig 1 4
Comparison between the results obtained with the 10 strains in the 6 laboratories

TABLE 5

Comparison of the Zone Diameters Observed in the 6 Laboratories
Disc Method Standard Medium
20 IU Penicillin

Strains	Laboratories 1	2	3	4	5	6	D	
Gc I	33.3[a] 5.0[b]	21.9 −6.4	28.6 0.3	27.9 −0.4	36.2 7.9	21.8 −6.5	28.3[c]	
Gc II	35.3 6.8	24.7 −3.8	34.6 6.1	23.7 −4.8	40.7 12.2	12.0 −16.5	28.5	
Gc III	36.3 4.0	29.6 −2.7	34.5 2.2	30.2 2.1	40.0 7.7	23.3 −9.0	32.3	
Gc IV	33.5 7.4	19.2 −6.9	27.7 1.6		31.6 5.5	18.3 7.8	26.1*	
Gc V	30.2 3.5	19.2 −7.5	31.2 4.5		32.5 5.8	20.5 6.2	26.7*	range 19.5
Gc VI	49.7 4.1	42.7 −2.9	50.2 4.6	40.3 5.3	53.9 8.3	36.8 8.8	45.6	
Gc VII	46.8 4.1	39.9 −2.8	46.7 4.0	36.8 −5.9	49.4 6.7	36.3 6.4	42.7	
Gc VIII	31.5 4.3	19.2 −8.0	27.8 0.6	37.1 9.9	31.8 4.6	15.7 11.5	27.2	
Gc IX	47.5 7.6	36.3 −3.6	40.2 0.3	41.7 1.8	43.0 3.1	30.7 −9.2	39.9	
Gc X	50.6 8.4	35.0 −7.2	43.4 1.2	41.5 −0.7	47.6 5.4	35.2 −7.0	42.2	
Average	39.5 5.5	28.8 −5.2	36.5 2.5	33.1* 0.9	40.7 6.7	25.1 8.9	34.0	

a Average of 3 zone diameters in mm See page 240
b Difference between 1 and 3
c Average of zone diameters for all laboratories
* Calculated by the method of ' missing plots

Differences in the laboratory levels might be eliminated by means of *reference strains*, which could be used to determine the level and to correct the individual results in accordance with this In the present investigation the averages for the 10 strains of gonococcus were used as an estimate of the laboratory level The resulting average differences are given in Tables 4–6, and in Figs 3 and 4 the laboratory levels are corrected Apparently, the inter laboratory variation of the corrected values was different for the various strains This is further elucidated in Table 7 in which the range of variation for each of the strains is presented, the corresponding values for Staph 209 P and Sarcina lutea are also included The range of variation of the individual strains should be compared with the total range of variation (in sensitivity) the latter is given at the base of the table With *the dilution method*, the average range of variation amounts to 18 per cent of the difference in sensitivity between the most sensitive and the least sensitive strain The corresponding values in *the disc method* are about 29 and 35 per cent for 2 and 20 IU, respectively Seen in relation to the range of sen

sitivity the variation in the individual results is greater with the disc method than with the dilution method This applies more obviously to the 20 IU-discs than to the 2 IU discs

TABLE 6

Comparison of the Zone Diameters Observed in the 6 Laboratories
Disc Method Standard Medium
2 IU Penicillin

Strains	Laboratories 1	2	3	4	5	6	D̄	
Gc I	23 7[a] >5 5[b]	<10 0 <-8 2	21 7 >3.5	20 1 >1 9	20 1 >1 9	13 7 > 4 5	<18 2[c]	
Gc II	26 2 >6 1	17 0 > 3 1	27 5 >7 4	15 9 >-4 2	28 2 >8 1	<6 0 <-14 1	<20 1	
Gc III	29 8 5 2	21 7 2 9	28 6 4 0	21 7 -2 9	28 3 3 7	17 2 -7 4	24 6	
Gc IV	20 3 >6 7	<7 0 <-6 6	18 2 >4 6		13 2 >-0 4	9 2 > 4 4	<13 6*	
Gc V	18 0 2 5	<8 1 -7 4	23 7 8 2		14 7 -0 8	13 0 -2 5	15 5*	range
Gc VI	43 6 5 1	37 2 -1 3	43 5 5 0	31 8 -6 7	43 3 4 8	31 5 -7 0	38 5	24 9
Gc VII	39 5 4 0	32 0 -3 5	40 0 4 5	31 1 -4 4	39 8 4 3	30 8 4 7	35 5	
Gc VIII	18 8 >4 6	<7 0 <-7 2	17 5 >3.3	21 0 >6 8	12 8 >-1 4	8 2 > 6 0	<14 2	
Gc IX	39 9 7 5	29 4 -3 0	33 4 1 0	33 0 0 6	32 2 -0 2	26 3 6 1	32 4	
Gc X	42 1 7 5	28 7 5 9	35 3 0 7	35 2 0 6	36 9 2 3	29.3 5 3	34 6	
Average	30 2 5 5	19 8 4 9	28 9 4 2	23 7* -1 0	27 0 2 2	18 5 6 2	<24 7	

[a] Average of 3 zone diameters in mm See page 240
[b] Difference between 1 and 3
[c] Average of zone diameters for all laboratories
* Calculated by the method of "missing plots"

The results obtained with the *R* showed substantially the same fea-
ti res as th

omewhat greater varia-

Thus, the two methods determined the relative sensitivities of the strains in the same way, irrespective of the medium, and apparently with somewhat smaller variation in the dilution method than in the disc method Seen in relation to the range of sensitivity the dilution method gave more uniform results than did the disc method

TABLE 7

Range of Variation of Results Obtained in the 6 Laboratories after Correction for the Laboratory Level Standard Medium

Strains	Dilution method		Disc method zone diameter in mm	
	N_{100}[1]	N_0	2 IU pen	20 IU pen
Staph aureus FDA 209 P	5.42	3.53	17.8	18.7
Sarcina lutea ATCC 9341	1.99	0.70	12.7	12.8
Gc I	1.10	1.16	6.2	4.6
Gc II	2.99	2.62	13.8	13.1
Gc III	0.80	0.51	3.9	4.0
Gc IV	0.99	0.92	4.4	3.6
Gc V	0.80	0.97	7.0	5.0
Gc VI	1.57	1.93	9.3	6.7
Gc VII	1.12	1.21	5.5	7.5
Gc VIII	1.43	2.07	11.4	13.6
Gc IX	1.77	0.93	5.2	6.3
Gc X	0.68	0.26	5.5	4.8
Average	1.33	1.26	7.2	6.9
Average range of sensitivity (Gc I–X)	6.99	7.14*	24.9§	19.5†
Average variation expressed in per cent of the average range of sensitivity (Gc) I–X)	19.0	17.6	28.9	35.4

[1] N_{100} = the highest dilution step in which complete inhibition is observed
* Table 4
§ Table 6
† Table 5

TABLE 8

Comparison of the Results Obtained in the 6 Laboratories Disc Method Standard Medium Difference in Zone Diameters in mm 20 IU Penicillin—2 IU Penicillin Gc Strains Arranged According to Sensitivity (N_0 Results from Table 4)

Strains	Laboratories 1	2	3	4	5	6	mm average (excl 4)	N_0 from Table 4
Gc IV	13.2	>12.2	9.5		18.4	9.1	>12.5	0.29*
Gc VIII	12.7	>12.2	10.3	16.1	19.0	7.5	>12.3	0.68
Gc V	12.2	>11.1	7.5		17.8	7.5	>11.2	0.85*
Gc I	9.6	>11.9	6.9	7.8	16.1	8.1	>10.5	1.77
Gc II	9.1	7.7	7.1	7.8	12.5	>6.0	>8.5	2.57
Gc III	6.5	7.9	5.9	8.5	11.7	6.1	7.6	3.85
Gc X	8.5	6.3	8.1	6.3	10.7	5.9	7.9	5.99
Gc IX	7.6	6.9	6.8	8.7	10.8	4.4	7.3	6.01
Gc VII	7.3	7.9	6.7	5.7	9.6	5.5	7.4	6.82
Gc VI	6.1	5.5	6.7	8.5	10.6	5.3	6.8	7.43

* Calculated by the method of missing plots

It is evident from the above that *the relation between the two methods was not the same in the different laboratories* However, the observations are not incompatible with the assumption that the relation be-

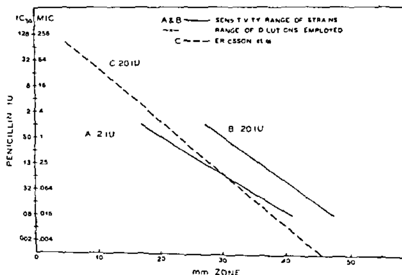


Fig 5

Regression curves showing the relation between MIC and IC_{50} (minimal inhibitory

TABLE 9

Experiment Performed at Bakteriologiska Institutionen Universitetet, Lund
By A B Laurell
Disc Method 2 and 20 IU Penicillin Effect of Variation of the Inoculum

Dilutions*	1 5		1 25		1 50		1 250	
Strain nos	Growth§	Zone mm	Growth	Zone mm	Growth	Zone mm	Growth	Zone mm
2 IU								
I	D	18 7	D	20 2	A	19.3	A	19 0
IV	D	11 0	D	10 3	A	10 5	A?	11 5
4289	D	33 0	D	33 3	D	32 8	A?	40 0
Average		20 9		21 3		20 9		23 5
Average difference			0 4		−0 4		2 6	
20 IU								
I	D	34 0	D	36 0	A	36.3	A	36 0
IV	D	30 3	D	30 8	A	31 5	A?	32.3
4289	D	40 5	D	43 0	D	44 0	A?	50 0
Average		34 9		36 6		37.3		39 4
Average difference			1 7		0 7		2 1	

* The undiluted suspension contained about 10^9 bacteria per ml
§ Growth D Dense A Appropriate S Sparse VS Very sparse

cent for labs 5 and 6 Thus it is unlikely that differences of this order would depend upon variation in the inoculum and, as mentioned above, the method of reading the results had also been different in the laboratories concerned

DISCUSSION AND CONCLUSION

The experiments showed that the relative sensitivity of the gonococcal strains was established with a substantial degree of uniformity by the two methods, whether the routine (R) or the standard (S) medium was employed However, the relation between the methods varied from one laboratory to the next This was presumably due mainly to variation in *the method of reading the results in the disc method* Different habits were encountered; in some of the laboratories the zone diameter was measured where the growth abruptly decreased from dense confluent to more granular growth, "outer zone" In other laboratories the more or less distinct limit between growth and no growth, the "inner zone', was measured At the conclusion of the experiments it became clear that "inner zones" had been read in lab 4 and to some extent in lab 6, whereas "outer zones" had been read in the other laboratories

The different results might also be due to *variation in the inoculum*, Ericsson (8) states that, for rapidly growing and less fastidious organisms, the zone without macroscopically visible colonies should be read and that the plates should be inoculated to obtain "dense growth but not confluent growth" With the gonococcus it is very difficult to obtain such an inoculum, the growth of the individual strains being variable By suspending cultures from plates (inoculated at varying times) it is difficult to keep the inoculum constant and owing to the varying degrees of viability turbidimetric measurements do not help When the experimental results were discussed it was discovered that generally the inoculum had not fulfilled the requirements described above Only in lab 5 had an attempt been made to follow the directions given by Ericsson, however, the result was growth which was often too sparse and zones which were relatively wide In supplementary experiments in which the inoculum was varied ,the density of the growth was also estimated, most frequently, two of the dilutions (5-fold steps) resulted in appropriate growth Accordingly, if only experimental results in which appropriate growth were obtained were to be accepted, the inocula would vary maximally by 25-fold from one laboratory to the next and, as a rule, the variation would not be more than 5 fold It is conceivable that the difficulty in keeping the inoculum constant might be overcome by shaking fluid cultures during incubation (9) This procedure is perhaps too laborious to be used routinely and fluid cultures are also difficult to check for contamination

Within a relatively broad range, *the dilution method is independent of the inoculum* (5) and in this method the readings can be rapidly

and easily performed In this case, also different methods of reading can be used and these may influence the determination of N_{50} In this respect it might be better to use N_{100} = the highest dilution step in which complete inhibition is observed (Corresponds to the minimal inhibitory concentration, MIC)

The relation between IC_{50} and MIC depends upon the distribution of the bacteria according to their sensitivities MIC corresponds to a concentration which is high enough to make the chance of the formation of even one colony very low With inocula of the order used in the present investigation (2–3 × 10) about one of each
[illegible] in contrast
(90–99 per
tested the
d by about
g to sensiti
vity was not very great As MIC will tend to be more influenced by variation in sampling than will IC_{50} there is no reason to prefer MIC to IC_{50} In other micro organisms it is possible that the distribution according to sensitivity is more
[illegible] oth MIC and IC_{50} might be of im
e 7 shows that the interlaboratory
In contrast to this *Sarcina lutea*
than for N_{50} presumably due to
the less abrupt decrease in growth with increasing concentration of the antibiotic

In spite of the above mentioned difficulties it is concluded that, *both methods are of practical value,* and quantitative determinations with the two methods will be positively correlated In a study, in which a number of theoretical possibilities were considered (1), it was demonstrated that from the results of the two methods a common value of sensitivity independent of the size of the inoculum can be derived

The dilution method requires the production of media containing various concentrations of antibiotic, which can only be stored for a limited period whereas *the disc method* permits long term storage of the discs in non specialized laboratories the disc method can be used with advantage

In order to "*translate* results obtained by the disc method into con centrations a comparison should be made with a dilution method This should be done in each laboratory with a large number of strains with sensitivities corresponding to the range encountered in the patients From time to time checks should be made to determine whether the observed correlation remains the same If the amount of antibiotic in the discs is altered a new comparison should be made

The curves reproduced in Fig 5 show an example of a translation', which is based on the results for 10 strains (average results of 3 determinations in each of 2 laboratories) which show a variation in MIC of about 7 dilution steps If, in each laboratory, 3 strains only were used to determine the curve the translation' from zone diameter to con centration would become less accurate the degree of the inaccuracy would depend upon the particular laboratory, especially upon the num ber of times tests were repeated in that laboratory The regression curves calculated for labs 1 and 3 *separately* showed, for the 10 strains,

cent for labs 5 and 6 Thus it is unlikely that differences of this order would depend upon variation in the inoculum and, as mentioned above, the method of reading the results had also been different in the laboratories concerned

DISCUSSION AND CONCLUSION

The experiments showed that the relative sensitivity of the gonococcal strains was established with a substantial degree of uniformity by the two methods, whether the routine (R) or the standard (S) medium was employed However, the relation between the methods varied from one laboratory to the next This was presumably due mainly to variation in *the method of reading the results in the disc method* Different habits were encountered; in some of the laboratories the zone diameter was measured where the growth abruptly decreased from dense confluent to more granular growth, "outer zone" In other laboratories the more or less distinct limit between growth and no growth, the "inner zone', was measured At the conclusion of the experiments it became clear that "inner zones" had been read in lab 4 and to some extent in lab 6, whereas "outer zones" had been read in the other laboratories

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Within a relatively broad range, *the dilution method is independent of the inoculum* (5) and in this method the readings can be rapidly

small effect because of the long lag and long generation times of the gonococcus. Furthermore they do not stand 20° C very well so that it would be necessary to use 24 hours prediffusion *before inoculation*. Consequently it seems reasonable to omit prediffusion completely, where from a practical point of view, prediffusion for 24 hours is considered as impossible or if the increased risk of contamination will play too great a role. By a long prediffusion period the concentration gradient in the medium becomes less steep and more stable and thereby the range of measurements is diminished. It might in this case be necessary to use several discs with different content in order to avoid the zones of the most sensitive strains being too large.

With gonococci it is very difficult to specify the inoculum and it is impossible to correct for the effect of this variation. The requirement of "dense but not confluent growth" is modified here to "confluent but granular growth" and if too sparse or too dense growth is obtained, the test should be repeated. By putting greater stress on the method of reading the zones it is hoped to reduce the inter-laboratory variation.

SUMMARY

10 strains of gonococci were selected so that the variation in their sensitivity to penicillin was the greatest possible; they were repeatedly tested by two methods (a dilution and a disc method) and with two different media, a common standard medium (S) and an individual routine medium (R). In both methods variations were observed both within the individual laboratory and between the laboratories.

Within a laboratory and within the range of sensitivity tested the variations of the two methods were substantially the same. The ranges of sensitivity were about seven 2-fold dilution steps and 20-25 mm in zone diameters. The inter-laboratory variation was mainly a difference in level (Figs. 1 and 2). In the dilution method the differences in level were small in relation to the range tested, whereas relatively large differences were found in the zone diameters measured by the disc method.

By a closer analysis of the results and of the experimental conditions especially in the disc method, it was found that differences in the method of reading the results and in the inoculum were very probably the main factors responsible for the observed deviations; both factor[illegible]
had [illegible]
perm[illegible]
speci[illegible]
diffi[illegible] the effect of variation of the inoculum. However
by stimulating certa[illegible]
o[illegible]
l[illegible]
u[illegible] improve the results through a correc

differences in translation of up to one dilution step With the 3 strains (nos 3, 5 and 7), which gave most uniform results in all laboratories, the deviation would not be much greater In practice, when "translating" (without repeated determinations) from mm to concentration one would expect a variation of 2 to 3 dilution steps It is not advisable to extrapolate the curve beyond the range tested Fig 5 shows the regression curve determined by *Ericsson* (5, 6, 7, 8) for 20 IU penicillin, this curve deviates from the common curve for labs 1 and 3, both with respect to position and slope This is not unexpected, as Ericsson's curve is based on results using a different medium and microorganisms with a higher growth rate and much shorter lag phase than that found in the gonococci Since the range of sensitivity tested is too small to determine whether or not the "line" is straight, it is preferred to describe the relation between zone and concentration as a "curve" and not as a "line"

The average level of sensitivity was somewhat different in the different laboratories and the deviation was, relative to the range, somewhat less with the dilution method than with the disc method (Tables 4, 5 and 6) For both, the difference from the average of all results were constant(or nearly so), making it possible to improve the results by means of a correction Similarly, a correction could be obtained by the use of *reference strains* with different sensitivities, strains with a small deviation from the corrected values should be preferred and strains nos 3, 5 and 7 could be used In contrast to this neither Staph 209 P nor Sarcina lutea are suitable they vary too much (Table 7)

The results may be further amended by a thorough specification of as many experimental conditions as possible media, agar (quality and content), thickness of plates, prediffusion time, inoculum and method of reading the results The experiments showed that minor variations in the composition of the medium and in the percentage of the agar played a relatively small role The thickness of the plates and the diffusion time were kept constant In supplementary experiments performed by *Laurell* (17), *Reyn* (23), *Wallmark* (30) and *Vogelsang* (31) a distinct, though not very marked, effect of prediffusion was demonstrated, but after 3 hours no constant level was found, at least not in strains giving large zones (5–8) Recently, *Thomsen* (28) has used long prediffusion times in different bacteria with varying lag periods and generation times He found that 1) "the difference between sizes of inhibition zones in strains with varied sensitivity will be marked and 2) the effect of variations caused by different growth rates and sizes of inoculum will be reduced' Based on this, he recommended a 24 hours' prediffusion with subsequent removal of the discs before the inoculation

In small laboratories with a varying amount of work it would be difficult to use such a long prediffusion time 3 hours' prediffusion *after inoculation,* as used in the present investigation, has a relatively

16 *Hirsch H A & Finland M* Susceptibility of gonococci to antibiotics and sulfadiazine A study of strains isolated at Boston City Hospital from cases of acute gonorrheal urethritis in males Amer J med Sci 239 41 1960
17 *Laurell A B* Personal communication 1961
18 *Medical Research Council Working Party* Resistance of gonococci to penicillin Lancet 7196 225 1961
19 *Perlrup A* Gonorrhoea in Denmark A survey of incidence over 82 years with comments on the reasons for the fluctuations noted Brit J vener Dis 37 115 1961
20 *Rantasalo I* Gonokokkien viljely ja nykyinen antibioottiherkkyys Duodecim 78 372 1962
21 *Reyn A Korner B & Bentzon M W* Effects of penicillin streptomycin and tetracycline on N gonorrhoeae isolated in 1944 and in 1957 Brit J vener Dis 34 227 1958
22 *Reyn A* Sensitivity of N gonorrhoeae to antibiotics Brit J vener Dis 37 145 1961
23 *Reyn A* Unpublished
24 Wilkinson A I
2
25 s Acta path et
26 ner Dis 37 138
1961
27 *Thayer J D Field F W Perry M J Martin F jr & Carson W* Surveillance
28
29
30
31
32
1921 pp 179 Disp
See also Nordisk Medicin 68 1491 1962 Nordiska kommitten for odling och resistensbestamning av gonokocker

tion from the average results from all the laboratories Thus, in future it will be possible to correct the individual results by using reference strains of known sensitivity

The disc method is preferable for routine use, but the results obtained by this method should be "translated" to concentrations by means of a regression curve, which has been determined by a series of tests with both methods This determination should be performed with strains covering the current range of sensitivity Despite the fact that both the inoculum and the method of reading the results have been specified in more detail, individual regression curves should ideally be prepared for each laboratory The course of the curves will depend upon a number of factors, of which the antibiotic contents of discs, prediffusion time, method of reading the results and inoculum are the most important Small differences in the composition of the medium did not play a large role, it is emphasized, however, that the composition of the routine and standard media was nearly the same It is probable, that differences in the contents of protecting (absorbing) and nutrient substances may have a very marked effect (24)

REFERENCES

1 *Bang J Bentzon M W & Reyn A* To be published
2 *Cradock-Watson J E Shooter R A & Nicol C S* Sensitivity of strains of gonococci to penicillin sulphathiazole and streptomycin Brit med J *1* 1091 1958
3 *Curtis F R & Wilkinson A E* A comparison of the *in vitro* sensitivity of gonococci to penicillin with the results of treatment Brit J vener Dis 34 70 1958
4 *Enfors W* A survey of female patients with gonorrhoea at St Gorans Sjukhus 1954 1958 Acta derm venereol *40* 393 1960
5 *Ericsson H Hogman C & Wickman K* A paper disc method for determination of bacterial sensitivity to chemotherapeutic and antibiotic agents Scand J clin Lab Invest *6* suppl 11 1954
6 *Ericsson H & Svartz Malmberg G* Determination of bacterial sensitivity in vitro and its clinical evaluation Antibiot and Chemother *6* 41 1959
7 *Ericsson H* Rational use of antibiotics in hospitals (Studies on laboratory methods and discussion of the biological basis for their clinical application) Scand J clin Lab Invest *12* suppl 50 1960
8 *Ericsson H* The paper disc method in quantitative determination of bacterial sensitivity to antibiotics From the Department of Clinical Bacteriology Antibiotic Section Karolinska Sjukhuset Stockholm 60 Sweden
9 *Gerhardt Ph & Heden C G* Concentrated culture of gonococci in clear liquid medium Proc Soc exp Med *105* 49 1960
10 *Gisslen H Hellgren I & Starck V* Incidence age distribution and complications of gonorrhoea in Sweden Bull Wld Hlth Org 24 367 1961
11 *Gjessing H C* Increasing failure rate after using the same preparation and dosage of penicillin in the treatment of gonorrhoea Brit J vener Dis 35 256 1959
12 *Gjessing H C* Tiltagende recidivhyppighet efter penicillinbehandling (samme preparat) for gonore T norske Lægeforen 79 82) 1959
13 *Gjessing H C* Gonoréproblemer i Norge særlig Oslo Nord Med *67* 119 1962
14 *Gjessing H C & Odegård K* Gonoré sensitivitet og behandling T norske Lægeforen *82* 572 1962
15 *Guthe T* Failure to control gonorrhoea Bull Wld Hlth Org 24 297 1961

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DECREASE IN FREQUENCY OF BIRTH IN MICE TREATED WITH ENZYMATIC DIGESTS OF DNA

By

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Received 20.x.62

The clonal selection theory of acquired immunity, once advanced though no longer championed in its original form by Burnet (*Burnet* 1959), not only requires but provides a fairly plausible mechanism for the elimination of auto-antibody forming cell clones in early development. In concordance with this theory natural tolerance could be dependent on elimination of certain antibody forming cell clones in embryonic development. However, the chemical basis for such an elimination mechanism is obscure. One possibility is that the death of auto-antibody forming clones may be caused by so called unbalanced growth, i.e. a phenomenon of dissynchronized nucleic acid and protein syntheses similar to "thymineless death" in bacteria (*Cohen & Barner* 1955). Thus it is conceivable that during early development, when DNA synthesis may occur at fairly high rates in most cells, unbalanced growth may be produced in those immunologically competent cells that in response to auto-antigens synthesize antibody protein at high rates. The observation that 6-mercaptopurine administered to adult rabbits together with an antigen will induce immunologic tolerance to this particular antigen but not to other antigenic substances (*Schwartz & Dameshek* 1959) is in line with such assumptions. The following experiments were undertaken with the thought that one might prevent such suspected unbalanced growth of certain antibody forming cells by stimulation of DNA synthesis during development. Direct studies with bacteria and more indirect observations with mammalian cells have indicated that DNA synthesis can be stimulated selectively with the aid of an enzymatic digest of DNA (*Braun* 1962) and this was therefore employed in attempts to cause possible auto-sensitization during early development.

EXPERIMENTAL

Mice from an inbred laboratory strain were used at an age of ca 6 weeks when they were highly fertile. To produce pregnancy fe-

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BRIEF REPORT

DEMONSTRATION OF THE RHEUMATOID FACTOR WITH HUMAN ANTI SHEEP CELL AMBOCEPTORS

By J Leikola K Aho and V-E Saris

It has been difficult to find anti Rh sera suitable for demonstrating rheumatoid factor (RF) activity There are sera which apparently do not sensitize the cells for the rheumatoid agglutination and with most of the reactive anti Rh sera only a low percentage of rheumatoid sera give positive agglutination reactions However, the need of a human antigen antibody system well suited for detecting the RF is evident

As the heterologous red cell is known to be a good immunogen and many different kinds of antibodies are formed against it we have immunized human subjects with sheep erythrocytes One or two intramuscular injections of 0 25 1 0 ml of washed packed cells were given Each of the six persons hitherto studied developed a relatively high titer of agglutinating makroglobulin antibodies The sera did not sensitize the cells for the rheumatoid agglutination However when the gamma globulin was separated from the other serum proteins by the DEAE cellulose column chromatography it appeared that all the sera contained a small amount of gamma globulin antibodies which were suitable for sensitization The agglutination experiments were carried out with the microscopic technique of *Harboe & Lundevall* (1959) The cells were sensitized with ½ ¼ of the minimum agglutinating dose (½–¼ MAD) A panel of six normal sera nine rheumatoid sera a pooled normal serum and a pooled rheumatoid serum was tested All the amboceptor sera gave positive reactions with the same sera which were reactive with the exceptional diagnostic' anti Rh serum Ripley The titers were definitely higher than those obtained with sheep red cells sensitized with homologous rabbit amboceptor (Waaler Rose test)

Two amboceptor sera (J L and K A) were studied in greater detail Absorption experiments with diphtheria toxoid human antitoxin precipitate were first carried out All RF activity measurable with these two amboceptors was absorbable to the precipitate and the titer decrease paralleled the decrease in the Waaler Rose test Next the effect of different sensitizing doses was studied It was observed than when weakly sensitized cells (1/16 1/32 MAD) were used some sera were still reactive in high titers whereas in some others no agglutination at all could be seen although the titers were equally high with strongly sensitized cells

Further experiments to explain the broader reactivity of the human anti sheep cell amboceptors as compared with the usual anti Rh sera are in progress

References Harboe M & Lundevall J Acta path microbiol scand 1959 45 357

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Supported by a grant from the *Sigrid Jusélius Foundation* and by research grant A 3026 from the *National Institute of Arthritis and Metabolic Diseases U S Public Health Service*

DEPARTMENT OF BACTERIOLOGY, KAROLINSKA INSTITUTET, STOCKHOLM 60, SWEDEN

DECREASE IN FREQUENCY OF BIRTH IN MICE TREATED WITH ENZYMATIC DIGESTS OF DNA

By

C.-G. HEDÉN, T. LINDAHL and W. BRAUN[1]

Received 20 x 62

The clonal selection theory of acquired immunity, once advanced though no longer championed in its original form by Burnet (*Burnet* 1959), not only requires, but provides a fairly plausible mechanism for, the elimination of auto-antibody forming cell clones in early development. In concordance with this theory, natural tolerance could be dependent on elimination of certain antibody-forming cell clones in embryonic development. However, the chemical basis for such an elimination mechanism is obscure. One possibility is that the death of auto-antibody-forming clones may be caused by so called unbalanced growth, i.e. a phenomenon of dissynchronized nucleic acid and protein syntheses similar to "thymineless death" in bacteria (*Cohen, Barner* 1955). Thus it is conceivable that during early development, when DNA synthesis may occur at fairly high rates in most cells, unbalanced growth may be produced in those immunologically competent cells that in response to auto antigens synthesize antibody protein at high rates. The observation that 6 mercaptopurine, administered to adult rabbits together with an antigen will induce immunologic tolerance to this particular antigen but not to other antigenic substance (*Schwarz & Dameshek* 1959) is in line with such assumptions. The following experiments were undertaken with the thought that one might prevent such suspected "unbalanced growth" of certain antibody forming cells by stimulation of DNA synthesis during development. Direct studies with bacteria and more indirect observations with mammalian cells have indicated that DNA synthesis can be stimulated selectively with the aid of an enzymatic digest of DNA (*Braun* 1962) and this was therefore, employed in attempts to cause possible auto-sensitization during early development.

EXPERIMENTAL

Mice from an inbred laboratory strain were used at an age of ca 6 weeks, when they were highly fertile. To produce pregnancy, fe-

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male and male mice were placed together, two and two in the same cage, for three days, whereupon the male mice were removed. The female mice were then treated, as shown in Tables 1 and 2, by injecting the indicated materials s.c. in the back every third day during gravidity. Each mouse usually received a total of 6 doses.

The DNA digest-containing mixture, which is not toxic for adult mice, consisted of 400 μg duponol-extracted and "sevag-treated" thymus DNA, 150 μg DNAase (Worthington, 1x cryst.), supplemented with 250 μg each of the four desoxynucleosides and desoxynucleotides, and 40 μg ADP per injection (=, supplemented DNA digest"). DNA was digested with DNAase in the presence of Mg^{++}-ions (0.01-M $MgSO_4$) before the nucleosides and nucleotides were added. Freshly distilled water was used as diluent. The test solutions were sterilized by filtration through membrane filters immediately after preparation, and stored at 4° C. They were regularly checked for sterility to avoid possible contamination with endotoxin-forming microorganisms.

RESULTS

In untreated control groups, the frequency of birth was high in all experiments. The administration of supplemented DNA digest produced a striking effect in some, but not in all, tests. The frequency of birth decreased, and some of the animals born to treated mice that yielded litters died two days after birth (Tables 1 and 2). The latter animals were of normal size at birth and did not differ visibly from he new-born animals in the control groups. These effects of DNA digest did not occur with mice that were treated with physiological saline, nucleosides + nucleotides, DNA alone, DNAase + nucleosides + nucleotides, or RNA + DNAase + nucleosides + nucleotides. DNA + nucleosides + nucleotides showed some effects in one experiment.

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Number of mice	Treated with	Size of individual litters
15	DNA + DNAase (Supplemented with deoxy nucleosides -tides and ADP)	0 0 0 0 0 0 0 0 0 0 0 7 8 8 10 (4T)
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15	DNA	0 0 3 3 3 4 6 8 8 9 9 9 9 9 10
15	Saline	0 0 0 3 4 4 5 5 5 5 7 8 9 10 11

(T) Indicates animals alive at birth but dead after two days

The reproducibility of the effects obtained with DNA digest depended to a large extent on the DNA preparation. Different batches of DNA

were found to vary greatly in activity some showing no activity at all when used after DNAase exposure and supplementation as outlined above We have obtained positive effects with a two year old preparation as well as with a freshly prepared duponol extracted DNA preparation Some of the active DNA's may have been partly denatured prior to enzyme treatment since they had been dissolved in distilled water during preparation However heat denaturation (100° C 15 minutes), prior to enzymatic digestion of an inactive DNA preparation did not produce any observable increases in biological activity That the variability of the results does in fact depend on the DNA preparation used was confirmed in an experiment in which digests were prepared at the same time from two different DNA preparations and were then tested under identical conditions (Table 2)

TABLE 2

Number of mice	Treated with	Size of individual litters
24	"active" DNA + DNAase + desoxynucleosides + desoxynucleotides	0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 (T) 3 4 5 7 8 9 (9T) 10 (10T) 10 10
19	"inactive" DNA + DNAase + desoxynucleosides + desoxynucleotides	0 0 0 0 0 1 2 3 5 6 6 7 7 8 8 8 9 9 10
19	RNA + DNAase + desoxynucleosides + desoxynucleotides	0 0 0 0 2 2 5 5 6 6 6 6 7 7 8 8 8 8 11
19	Saline	0 0 0 4 4 6 7 7 7 8 8 8 8

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Differences in biological activity between different DNA digests have also been observed in microbiological test systems (*Braun* 1962) and differences in presumably identically extracted DNA preparations also have been noted in immunological tests (*Paleruk Plescia & Braun* 1961)

DISCUSSION

Due to the variability in effectiveness of different DNA digest preparations in the here described tests with pregnant mice no detailed pathological investigations were performed on animals from litters which died two days after birth The variability encountered in different experiments also precludes a detailed discussion of the suggestive data so far obtained Instead we prefer to postpone such a discussion until more conclusive experiments can be perfor[illegible]

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male and male mice were placed together, two and two in the same cage, for three days, whereupon the male mice were removed The female mice were then treated, as shown in Tables 1 and 2, by injecting the indicated materials s c in the back every third day during gravidity Each mouse usually received a total of 6 doses

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The reproducibility of the effects obtained with DNA digest depended to a large extent on the DNA preparation Different batches of DNA

THE ENTEROVIRUS DEPARTMENT, STATENS SERUMINSTITUT, COPENHAGEN, DENMARK

NEUTRALIZATION OF VISNA VIRUS BY HUMAN SERA

By

HALLDOR THORMAR[1] and HERDIS VON MAGNUS

Received 28 ix 62

In a previous paper (12) the results of neutralization tests with human sera against visna virus were reported. These preliminary experiments included sera from healthy individuals as well as sera from patients suffering from multiple sclerosis (MS) The reason for testing the sera of these patients against visna virus was the apparent similarity between visna infection in sheep and the human demyelinating diseases as pointed out by *Sigurdsson & Palsson* (8)

In the preliminary study, visna virus (about 100 $TCID_{50}$) was found to be neutralized by MS sera in dilutions up to 1 16 However, it was not ascertained whether there was a difference in the neutralizing activity of MS sera and of normal human sera, and the nature of the neutralizing substance was not studied

The present paper reports the results of neutralization tests carried out with human sera in a larger number than tested in the preliminary study Both normal sera and sera from patients with MS were examined

MATERIAL AND METHODS

Tissue culture —Serially propagated cell cult [illegible] with 1 per cent sheep serum Before changing the cultures to the maintenance medium they were washed with Hanks' salt solution to remove neutralizing substances present in the calf serum (12)

Virus Visna virus strain K487 in its 40th TC passage was employed The virus was titrated in tenfold serial dilutions in roller tubes The batch of virus used in the present experiments had an infectivity titer of about $10^{4.5}$ per 0 1 ml

Sera Sera from patients with m [illegible] s were kindly supplied by the [illegible] hagen, and by the Neurological [illegible] The sera derived from patients [illegible] phases of the disease

[1] Present address Institute for Experimental Pathology, University of Iceland Keldur Reykjavik Iceland

The authors are [illegible] partment Mun [illegible] of the Neurolo [illegible] supply of sera [illegible] rosis

test systems in the laboratory of one of us (W B) In the meantime, however, we felt it to be of value to place the available observations on record

SUMMARY

Treatment of mice with enzymatic digests of DNA during pregnancy in some cases gave rise to a decrease in frequency of birth The activity of the digested DNA apparently varied with different DNA preparations

REFERENCES

Braun W Symposium on Biological Interactions in Normal and Neoplastic Growth Little Brown & Co 1962

Burnet F M The clonal selection theory of acquired immunity Cambridge University Press 1959

Cohen S S & Barner H D The Death of Bacteria as a Function of Unbalanced Growth Pediatrics *16* 704 708 1955

Palczuk N C Plescia O J & Braun W Studies on DNAase sensitive Antigens of Brucella abortus by Complement Fixation Proc Soc Exp Biol Med *107* 982 986 1961

Schwartz R & Dameshek W Drug induced Immunological Tolerance Nature *183* 1682 1683 1959

normal sera—To compare the content of heat stable neutralizing substance in MS sera and in normal human sera a total of 109 heated sera of each kind was tested against visna virus The results of two experiments comprising all the sera are shown in Table 2 It can be seen that almost an equal number of positive sera was found in each group The number was somewhat lower when a larger virus dose was employed The distribution of neutralizing titers was almost identical in the two groups

TABLE 1

Effect of Heating to 56° for 30 Minutes on the Neutralizing Activity of Human Sera against Visna Virus Eight Sera Heated and Unheated Were Incubated with Virus a) at 22° for 24 Hours or b) at 37° for 18 Hours before Inoculation into Tissue Cultures*

		Number of sera with titer				Total positive	Titer of antiserum no 5383
		< 4	4	8	> 16		
22° / 24 hrs	Not heated	4	3	1	0	4	
	Heated	8	0	0	0	0	180
37° / 18 hrs	Not heated	0	0	1	7	8	
	Heated	3	1	4	0	5	256

* 30 $TCID_{50}$ per 0 1 ml of mixture

TABLE 2

Neutralizing Substance against Visna Virus Compar… of Titers in Sera from … …an Sera The Sera Were … Virus per 0 1 ml of

		No of sera tested	Negative in dil 1 4	Number of sera positive in titer					Total positive
				4	6	8	11	≥ 16	
a)	MS	57	7	11	14	9	7	9	50
	Normal	57	6	11	7	15	14	4	51
b)	MS	52	16	11	14	8	1	2	36
	Normal	52	13	17	12	7	3		39

From the above experiments it was concluded that small amounts of the heat stable neutralizing substance against visna virus were found widely distributed in adult human sera and that there was no …

… *sera from children*—In an attempt to characterize the heat stable substance it was first studied whether there was an age correlated difference in its occurrence in normal human sera Table 3 shows the neutralizing titers found in heated sera from 79 children ranging in age from 2 months to 11 years, compared with the titers found in sera from adults Positive sera were found in all of

Normal human sera were obtained by kind permission of blood donors at the Blood Bank of Statens Seruminstitut through the courtesy of *Dr P Ejby Poulsen* The donors represented the same age groups, ranging from 18 to 65 years as the patients from which serum was received Sera from children were kindly supplied by Dr Inger Petersen of the Enterovirus Department, Statens Seruminstitut

Neutralization tests—Serial twofold dilutions of the human sera were prepared in medium 199 and 0 3 ml of each dilution were mixed with an equal volume of visna virus diluted in medium 199 to an estimated concentration of 50 $TCID_{50}$ per 0 1 ml of serum virus mixture The pH of the mixtures was adjusted to 7 3–7 5 by gassing the tubes with 5 per cent CO_2 in air After incubation the serum virus mixtures were inoculated in 0 1 ml amounts into roller tube cultures using two tubes per serum dilution The roller tubes were incubated at 37°,[1] first in a roller drum for two days and subsequently in stationary racks They were observed for cytopathic changes at intervals and were discarded after 14 days Neutralizing titers were calculated by the method of *Karber* (5) and are expressed as the reciprocal of the highest initial serum dilution which completely suppressed cytopathic changes in 50 per cent of the inoculated tubes

In each experiment the virus dilution employed was mixed with a 1 8 dilution of normal sheep serum to serve as a virus control The virus control was titrated at the end of the incubation period simultaneously with the inoculation of the serum virus mixtures Visna antiserum no 5383 with a known neutralizing titer (11) was included as a positive control in all experiments This serum was kindly supplied by *Dr Pall A Palsson* of the Institute for Experimental Pathology, Keldur Iceland

Prior to inoculation into tissue cultures the serum virus mixtures were incubated at 37° for 18 hours except otherwise stated These conditions of incubation had previously been found to result in a maximal neutralization of visna virus by antiserum with only a slight spontaneous inactivation of virus at the serum concentrations employed (11)

RESULTS

Effect of heating the sera to 56° for 30 minutes—In pilot experiments carried out with human sera incubated with visna virus at 22° for 24 hours prior to inoculation into roller tubes, neutralizing titers varying from less than 4 to at least 16 were observed in unheated sera Upon heating the sera to 56° for 30 minutes before mixing with the virus, the titers decreased to below 4 However, in serum dilution 1 4 the viral activity was markedly reduced, indicating some neutralization of virus by heated sera In an attempt to increase this neutralizing effect, serum-virus mixtures were incubated at 37° for various periods of time before inoculation into roller tubes Table I shows the results of one experiment comparing the neutralizing activity in unheated and in heated sera incubated with virus at 22° for 24 hours or at 37° for 18 hours It can be seen that by raising the incubation temperature the titers were considerably increased, both in unheated and in heated sera In the latter, about half of the sera neutralized the virus in dilution 1 4 or higher This experiment showed that in addition to the "heat-labile" substance the human sera contained a "heat-stable" substance which neutralized visna virus during incubation at 37° for 18 hours The term "heat-stable" will in the following sections be used to denote the ability to resist heating to 56° for 30 minutes

Comparison of heat-stable neutralizing substance in MS sera and in

1 All temperatures given in centigrade

8 *Sigurdsson B & Palsson P A* Visna of Sheep A slow demyelinating infection Brit J Exp Path 39 519 528 1958
9 *Sigurdsson B Thormar H & Palsson P A* Cultivation of visna virus in tissue culture Arch ges Virusforsch *10* 368–381 1960
10 *Thormar H* Stability of visna virus in infectious tissue culture fluid Arch ges Virusforsch *10* 501 509 1960
11 *Thormar H* Neutralization of visna virus by antisera from sheep J Immunol 1963
12 *Thormar H & Sigurdardóttir B* Growth of visna virus in primary tissue cultures from various animal species Acta path et microbiol scandinav 55 180–186 1962
13 *Utz J* Effect *in vitro* of specific lipid fractions of animal sera on psittacosis virus Proc Soc Exp Biol Med *69* 186–189 1948
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In an attempt to distinguish between these two possibilities, the neutralizing substance was studied with regard to a few characteristics considered to be typical of true antibodies (4, 6) The results, showing a) absence of neutralizing activity in the gamma globulin preparation tested, b) an apparent absence of an age pattern indicative of immune response, and c) resistance of the substance to heating at 80°, seem to indicate that the neutralizing substance described in the present study was not a true antibody

Apart from the above evidence against an antibody status of the neutralizing substance, its nature remains unknown The resistance to treatment with periodate indicates that it is not a mucopolysaccharide (3, 7) Furthermore, preliminary experiments carried out to study whether we were dealing with viral inactivating lipids (1, 13, 14) were negative

SUMMARY

The majority of human sera studied in neutralization tests against visna virus were found to contain substances which neutralized the virus These seemed to be of two kinds, one which was inactivated by heating to 56° for 30 minutes, and another which was not affected by this heating The latter substance was most easily detected after incubation with virus at 37° for 18 hours It was found as often in sera from normal blood donors as in sera from patients with multiple sclerosis It was found in sera from children ranging in age from 2 months to 11 years and no typical age pattern could be demonstrated The heat stable factor was not found in preparations of human gamma globulin and aparently it was not destroyed either by heating to 80° for 30 minutes, or by treatment with trypsin, periodate or ethyl ether It is concluded that the heat-stable substance most likely is a non-specific inhibitor of visna virus

REFERENCES

1 *Casals J & Olitsky P K* Inactivation of certain neurotropic viruses *in vitro* by serum lipids Science *106* 267 268 1947
2 *Fazekas de St Groth S & Graham D M* Modification of virus receptors by metaperiodate II Infection through modified receptors Aust J Exp Biol Med Sci 27 83–98 1949
3 *Hirst G K* The nature of the virus receptors of red cells I Evidence on the chemical nature of the virus receptors of red cells and of the existence of a closely analogous substance in normal serum J Exp Med 87 301 314 1948
4 *Karzon D T* Studies on a neutralizing antibody against canine distemper virus found in man Pediatrics *16* 809 818 1955
5 *Karber G* Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche Arch f exp Path u Pharmakol *162* 480 514 1931
6 *Klein M* The significance of human antiviral neutralizing substances in animal sera Ann N Y Acad Sci *70* 362 368 1958
7 *Mandel B & Racker, E* Inhibition of Theiler s encephalomyelitis virus (G D VII strain) of mice by an intestinal mucopolysaccharide II Purification and properties of the mucopolysaccharide J Exp Med *98* 417–426 1953

8 *Sigurdsson B & Palsson P A* Visna of Sheep A slow demyelinating infection. Brit J Exp Path *39* 519 528 1958
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SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

I. *Plan. Material*

By

TOR OMLAND

Received 6.x.61

Haemophilus influenzae (Pfeiffer's bacillus) has been the subject of great interest in medical bacteriology not only earlier, before the hypothesis of the organism as primary causal agent in epidemic influenza was finally abandoned, but also during recent times when it has been recognized as sole pathogenic agent in certain conditions of a septicaemic nature, notably meningitis and septicaemic laryngitis. It seems also increasingly clear that it plays an important role in a series of less dramatic infections of the respiratory tract and its adnexa.

The earlier literature on H. influenzae has been thoroughly reviewed by several authors (*Scott* 1929, *Engbæk* 1949, *Wilson & Miles* 1955, pp. 896–925, *Alexander* 1958, *Zinnemann* 1960), and readers are referred to bibliographies found in these works.

The finding that certain conventional serological procedures, such as agglutination, generally yielded inconsistent results was the object of a series of studies (*Park, Williams & Cooper* 1918, cit. *Wilson & Miles* 1955, p. 909, *Anderson & Schultz* 1921, *Yabe* 1921, *Krah* 1930, *Yagi* (= *Iwata*) 1935, 1938, *Platt* 1937). Such results pertained to what was customarily called R strains. The so-called S strains, mostly isolated from cases of meningitis, on the other hand presented a more orderly picture (*Povitsky & Denny* 1921, *Rivers & Kohn* 1921, *Pittman* 1929, 1931, *Fothergill & Chandler* 1936, *Wilkes-Weiss* 1937, *Engbæk* 1939, 1950).

More recently the line of work on the serology of H. influenzae has been directed towards immunochemical questions, especially the problem of the chemical composition of the different specific soluble substances (*Dingle & Fothergill* 1939, *Alexander & Heidelberger* 1940, *MacPherson, Heidelberger, Alexander & Leidy* 1946, *MacPherson* 1948).

Zamenhof, Leidy, Fitzgerald, Alexander & Chargaff (1953) showed that the capsular type-specific substance of type b was a polyribophosphate and thus a polymer of somewhat unusual composition. *Zamenhof & Leidy* (1954) reported further investigations on polyribo

phosphate from H influenzae type b and on poly sugarphosphates from type a and type c *Rosenberg Leidy Jaffe & Zamenhof* (1961) published the results of investigations on the type specific substances of type e and type f They showed that the type e substance in contrast to the other type specific substances contained two monosaccharide components glucosamine and a hexose but no phosphate The type f substance apparently was composed of disaccharide units of N acetyl glucosamine linked through phospho diester linkages

The unique composition of the specific capsular substance of type e had been indicated earlier by *Williamson & Zinnemann* (1951 1954) They reported the occurrence of two distinct type specific components as demonstrated by serological technique and discussed a possible analogy with the phase variation in Bordetella pertussis

PURPOSE AND PLAN OF THE INVESTIGATION

At this stage some remarks must be made on terminology Unless otherwise stated the termini mucoid (M) smooth (S) and rough (R) will be used in this study in describing morphological variants This is in agreement with the principles followed by *Chandler Fothergill & Dingle* (1939) who postulated a range of variation from M through S to R characterizing all serologically typable (Pittman types) meningitic strains as M strains the common respiratory strains as S strains and as R strains only strains with a typical rough morphology The mentioned use of termini is in discordance with the practice adopted by *Pittman* (1929 1931) and others who preferred to call the morphologically mucoid variant smooth or S *Wilson & Miles* (1955 p 323) proposed to reserve the words mucoid smooth and rough as morphological descriptives exclusively and to use the letters M S and R as designations of antigenic variants thus dissociating these two sets of termini from each other This system appears somewhat artificial and will not be adhered to in the subsequent studies

The following points are fundamental in the discussion leading to working hypotheses and plans

1 *The M–S–R Variation as a General Phenomenon in Bacteriology*

In man [illegible]

c n t tut

Her results were confirmed by *Platt* (1937), *Chandler, Fothergill & Dingle* (1939). The latter authors used, however, another terminology in the description of variants (see above).

According to these results H. influenzae would fit well into the general M-S-R variation scheme.

2) *The Shiny Appearance of the Smooth Colony*

This type of colony is remarkably lustrous, and scarcely deserves the descriptive 'rough' as used in the Pittman terminology (*Chandler, Fothergill & Dingle* 1939).

3) *The Presence of Type Specific Capsular Substance in Experimentally Produced Variants*

MacPherson (1948) has shown that considerable amounts of type specific substance can be extracted from intermediate forms during experimental conversion from M to R, even at a stage when a capsule can no longer be detected by other methods.

4) *The Apparent Virulence of the Smooth Form*

As already mentioned in the introductory remarks it seems that H. influenzae plays an important role in infections of the respiratory tract, especially such as rhinopharyngitis, sinusitis, otitis media and bronchitis. This pertains also to the commonest form, i.e. the smooth variant (*Schafer & Martin* 1959, *Zinnemann* 1960, *Edlund, Richtnér, Tunevall & Wasserman* 1960).

These points would serve to illustrate the paradoxical situation concerning the serology of the S-form of H. influenzae, and represent the basis of the following *main questions*:

Does the S-form of H. influenzae possess superficial antigen(s) which, in conformance with general bacteriological principles, would explain better its colonial morphology, and apparent pathogenicity?

Is the variation pattern in H. influenzae reflected in changes in the equipment of more deeply situated, or more firmly bound, antigens (in this study called somatic antigens)?

The *purpose* of this study would then be to investigate:

(a) the occurrence of superficial antigens, especially of type specific, capsular nature, in a collection of H. influenzae strains (and a limited number of strains of other species).

(b) the occurrence of somatic antigens in selected strains of the material.

The following general *plan* has been established for the study:

(a) Basic characterization of the material of strains.

(b) Examination of the type specific antigens by means of the capsular swelling method, and, after adaptation of the technique, by means of the gel precipitation method.

(c) Evaluation and comparison of the two sets of results.

(d) Search for other antigens by means of the gel precipitation and the immunoelectrophoresis method.

COLLECTION AND CLASSIFICATION OF THE MATERIAL

The material consists of Haemophilus strains of the following categories

1 ... let Oslo
2 ... ns of practitioners ... ouring districts

The collection of strains has continued from ... February 1961 and has included a total of 212 strains

The isolations have been made during the ordinary routine mainly on the basis of characteristic morphological and cultural properties At this stage the most ... Miles 1955 ... phenomenon ... a staphylo... our (*Schaub* ... necessary the secondary culture has been been laid on ordinary blood agar ... on a practical basis without any preconceived ... not been within the first hundred strains ... that during this period the majority of strains of Haemophilus occurring in the laboratory has been included The second hundred strains are approximately evenly distributed on the remaining period During this time the selection has been slightly more concentrated on a) mucoid strains b) haemolytic strains

The ... antages and ... *1938* *Heckly* ... 1958) A num... order to reduce to a minimum the number of necessary transfers during the work. No strain used in the study has been subcultured more than five times

Whereas the preliminary examination of the strains has been done according to the principles mentioned above, the final diagnosis has included several other properties, and has essentially been made in accordance with *Alexander* (1958) In the following an account shall be given of the single properties used for classification

1) *Morphology*

a) Colonial morphology *Circular*, opaque to translucent colonies with entire edge and a more or less *dense center* *In the S-forms* the edge was often splayed out and the center a little raised The diameter has been estimated at 0 2 to 2 0 mm after 18–20 hours' incubation at 37° C This pertains to the diameter of the largest colonies as near as possible to the staphylococcus streak Distinction has been made between mucoid (M), smooth (S), *and rough (R)* appearance of colonies according to *Chandler, Fothergill & Dingle* (1939) The presence or absence of iridescence of the colonies on Levinthal agar (*Levinthal* 1918 *Alexander* 1958) has *been* noted (*Scott* 1929, *Pittman* 1929) This character has been observed *on 18–20* hours' cultures with an arrangement of light as shown in Fig 1

It was necessary as a rule to view the plate from different angles within the angle v to find the position of maximum iridescence It was

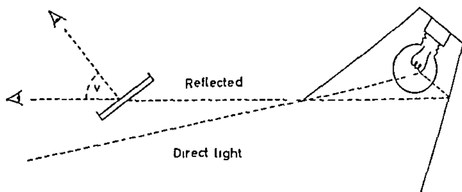


Fig 1
Arrangement for examination of iridescence

also often advantageous to vary the angle at which the plate was held in the ray of light (*Engbæk* 1949)

b) Microscopical morphology The well known polymorphism within the genus Haemophilus from species to species, or from strain to strain, as well as within the same strain under different conditions, made a microscopical classification difficult Two main groups, however, have been distinguished The first group consisted of short, coccobacillary forms with estimated dimensions of 0 3–0 5 μ × 1 μ Often these forms were mingled with longer bacillary forms of the same general width In some strains bacillary forms or even filaments were prevalent The second group consisted of coarse bacillary forms, some times up to 1 μ broad and several μ long The typical H influenzae and for the most part also H parainfluenzae are examples of the first group, H parahaemolyticus (and the two strains of H haemolyticus) of the second group These findings are in good agreement with the descriptions found in the literature (*Rivers & Leuschner* 1921, *Loewenthal & Zurukzoglu* 1928, *Smith* 1931, *Wilson & Miles* 1955, pp 897 899 and pp 917 918 *Alexander* 1958)

The microscopical morphology has been examined both in wet mounts by ordinary and by phase contrast illumination, and after Gram staining Dilute carbol fuchsin has been found most satisfactory as a counterstain (*Pfeiffer* 1893), but safranin has also been used The absence of spores and of active motility has been noted

2) *Nutritional Requirements*

a) Aerobiosis All strains grew well under atmospheric oxygen tension

b) Requirements of growth factors The need of the so-called X-factor (haematin or related compounds) and the V-factor (di or triphosphopyridine nucleotide) is well established (*Davis* 1921, *Fildes* 1921, 1922, 1924, *Thjotta & Avery* 1921 *Lwoff & Lwoff* 1937) The determination of growth factor requirements has been carried out according to a procedure described by *Pickett & Stewart* (1953) It is based on the observation that catalase producers (*e g* Sarcina lutea)

supply both X- and V-factor, whereas catalase non-producers (e g Streptococcus faecalis) provide only V-factor during growth The growth requirements of a Haemophilus strain may thus be observed by registering the presence or absence of satellitism near colonies (or streaks) of Sarcina lutea and Streptococcus faecalis, when grown on a medium otherwise devoid of the mentioned growth factors As a medium for such experiments the Heart Infusion Broth (Difco) with 1 5 per cent agar has been used

According to the principles referred to above a classification of the strains has been made, and the results are summarized in Table 1

TABLE 1
Distribution of the Material of Strains According to Species

Species	Number of strains	
H influenzae	163	196
H parainfluenzae	7	
	2	
	24	
.	13	
	3	
Total	212	

Table 2 shows the distribution of strains according to origin of the pathological material, and bacterial species It also shows the number of isolations of H influenzae made in pure culture It is seen that a majority of the strains of H influenzae has been isolated from the upper respiratory tract and from cerebrospinal fluid

TABLE 2
Distribution of Strains According to Origin of Pathological Material and Bacterial Species

Origin	H infl	H para infl	H haem	H para haem	Totals
Nose	95 (19)	3	1	8	107
Throat	3 (0)	1		10	14
Sputum	7 (0)		1	6	14
Bronchus	5 (0)				5
Sinus	24 (15)				24
Ear	10 (7)	1			11
Conjunctiva	2 (1)	1			3
Vagina	1 (1)	1			2
Cerebrospinal fluid	14 (14)				14
Blood	2 (2)				2
Totals	163 (59)	7	2	24	196

The figures in brackets represent the number of primary isolations in pure culture (shown only in H influenzae)

As concerns H influenzae it is also seen that there is a greater tendency towards isolation in pure culture from the upper parts of the respiratory tract and adnexa (nose, sinus, and ear) than from the lower parts (throat and bronchi), where H influenzae has been found in mixed culture in all cases In cerebrospinal fluid and blood all isolations have been made in pure culture

Table 3 presents the distribution of strains according to species and category of variation (colonial morphology)

TABLE 3

Distribution of Strains According to Species and Category of Variation (Colonial Morphology)

Species	Mucoid (M)	Smooth (S)	Rough (R)	Totals
H influenzae	60	102	1	163
H parainfluenzae		7		7
H haemolyticus		2		2
H parahaemolyticus	3	15	6	24
Totals	63	126	7	196

The table shows that out of a total of 163 H influenzae strains 102 are S-strains, in spite of the fact that the M-form was overrepresented during the collection of the material (see above)

The incidence of mucoid growth and iridescence, and the coincidence of these two properties, are shown in Table 4

TABLE 4

Incidence and Coincidence of Mucoid Growth and Iridescence in two Haemophilus Species

Species	Number of strains showing		
	Mucoid growth + iridescence	Mucoid growth alone	Iridescence alone
H influenzae	51	9	2
H parahaemolyticus		3	

It should be pointed out that the examination for iridescence has been made after 18 20 hours incubation not after about 8 hours as recommended by *Alexander* (1958), see also *Engbæk* (1949) The table shows that there is no complete correlation between iridescence and mucoid growth under these conditions

Under otherwise similar conditions (as near as possible to the staphylococcus streak on blood agar) there was a definite relationship between category of colonial variation (M or S) and estimated diameter of the colonies In the majority of M-strains the diameter was between

1 and 1.5 mm, whereas in most of the S-strains the diameter was between 0.5 and 1 mm. On the average the diameter of the M colonies was nearly twice the diameter of the S-colonies.

The distribution of the morphological variants according to isolation in pure, respectively mixed, culture is shown in Table 5.

TABLE 5

Distribution of H. influenzae Strains According to Category of Variation and State of Primary Isolation (Pure or Mixed Culture)

Category of variation	Number of strains		
	Pure culture	Mixed culture	Totals
Mucoid (M)	25	35	60
Smooth (S)	34	68	102
Rough (R)		1	1
Totals	59	104	163

It is noted that one third of the smooth strains have been isolated in pure culture.

The whole material has been screened by direct haemagglutination tests (human type O erythrocytes) to sort out eventual strains of H. aegyptius (*Breed, Murray & Smith* 1957, *Wilson & Miles* 1955, pp. 918–919). No such strains have been found.

DISCUSSION

It has been shown by many workers that the S forms are the variants most frequently isolated from normal as well as from pathological conditions. Thus *Straker* (1945) described the results of serological typing of meningitic strains and strains from the respiratory tract in school boys, children in an orphanage, and healthy adults. Even though she was able to isolate a considerable number of M strains (especially among the children), the great majority of strains were S-strains.

Engbæk (1949), in a serological study of H. influenzae, found only a minor part of his collection of strains from pathological processes to be typable M-forms.

It is thus an accepted fact that S-forms are the variants most frequently isolated, also when pathological conditions are considered separately. While the most dramatic clinical pictures are caused by M forms, they are numerically surpassed by more banal conditions such as sinusitis, otitis media, rhinopharyngitis, and bronchitis, conditions in which S forms are predominant.

The present material serves as a good illustration of the preponderance of the S-form. Table 3 shows that out of a total of 163 H.

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Dingle J H & Fothergill L D The isolation and properties of the specific polysaccharide of type b Hemophilus influenzae J Immunol *37* 53 63 1939

Fillund G Richtner N G Tunevall G & Wasserman J The significance of Haemophilus influenzae in chronic respiratory disease as elucidated by serological tests Acta Otolaryng (Stockholm) *52* 316 320 1960

Fngbæk H C Undersøgelser over Pfeiffers bacil Serologi Infektionens forløb hos mus Behandling af Pfeiffermeningitis Nyt Nordisk Forlag – A Busck København 1949 245 pp

Fngbæk H C Pfeiffer s bacillus Serological studies with special reference to capsular antigens Acta path et microbiol scandinav *27* 378 387 1950

Fildes P The nature of the effect of blood pigment upon the growth of B influenzae Brit J Exp Path *2* 16 25 1921

Fildes P The nature of the action of potato upon the growth of B influenzae Brit J Exp Path *3* 210–214 1922

Fildes P The growth requirements of haemolytic influenza bacilli and the bearing of these upon the classification of the related organisms Brit J Exp Path *5* 69 74 1924

Flosdorf E W & Mudd S Procedure and apparatus for preservation in lyophile form of serum and other biological substances J Immunol *29* 389–425 1935

Flosdorf E W & Mudd S An improved procedure and apparatus for preservation of sera microorganisms and other substances—the cryochem process J Immunol *34* 469–490 1938

Fothergill L D & Chandler C A Observations on the dissociation of meningitis strain of H influenzae J Immunol *31* 401 415 1936

Grassberger R Beitrage zur Bakteriologie der Influenza Z Hyg Infektionskr *25* 453 475 1897

Heckly R J Anderson A W & Rockenmacher M Lyophilization of Pasteurella pestis Appl Microbiol *6* 255 261, 1958

Iruka A (= Yagi A) Weitere serologische Einteilung von Influenza bazillen Z Immunitaetsforsch *95* 312 318 1939

Krah F Zur Serologie und Typeneinteilung der Influenzabazillen Zbl Bakt (Orig) *116* 101 113 1930

Leuinthal W Bakteriologische und serologische Influenzastudien Z Hyg Infektionskr *86* 1–24 1918

Loewenthal W [illegible]

Lwoff [illegible]

Mac [illegible]

MacPherson C [illegible] saccharides [illegible] 207 219 19[illegible]

Pfeiffer R Die Aetiologie der Influenza Z Hyg Infektionskr *13* 357 386 1893

Pickett M J & Stewart M A Identification of hemophilic bacilli by means of the satellite phenomenon Amer J Clin Path 23 713 715 1953

Pittman M The S and "R forms of Hemophilus influenzae Proc Soc Exp Biol Med *27* 299 301 1929

Pittman M Variation and type specificity in the bacterial species Hemophilus influenzae J Exp Med *53* 471–492 1931

Platt A F A serological study of Haemophilus influenzae J Hyg (London) 37 98 107 1937

Povitsky O R & Denny H T W Further studies on grouping of influenza bacilli with special reference to permanence of type in the carrier J Immunol *6* 65 80 1921

Rivers T M & Kohn L A The biological and the serological reactions of influenza bacilli producing meningitis J Exp Med 34 477 494 1921

Rivers T M & Leuschner E L Hemolytic influenza bacilli Bull Johns Hopkins Hosp 32 130 132 1921

influenzae strains 102 were S-strains, and from Table 5 it is seen that 34 (about one third) of these strains have been recovered in pure culture, thus supporting the assumption of a more direct causal connection between the S-form and the pathological process at hand It should be stressed, however, that isolation, even in pure culture, does not per se allow definite conclusions to be drawn as to the aetiological rôle played by the organism in that particular case

It is interesting to note that there is no complete parallel between mucoid growth and iridescence in H influenzae (Table 4) This may be due partly to the use of the long incubation of 18–20 hours The phenomenon of iridescence has been explained by assuming a particular spatial arrangement of the single cells in the colony (*Engbæk* 1949) It is possible that this arrangement persists only for a certain period during the growth, while the mucoid property in general remains more or less permanent

The existence of iridescence alone without mucoid growth is more difficult to interpret The characterization of a colony as mucoid must necessarily be arbitrary, based on quantitative rather than qualitative criteria It is thus possible that the two strains demonstrating iridescence alone, have reached the necessary spatial arrangement of cells while still possessing so small amounts of capsular substance as to lead to their grouping in a non-mucoid category

SUMMARY

Based upon a short review of earlier literature the relationship between antigenic structure and morphological variation in H influenzae is discussed, concentrating on problems connected with the smooth (S) variant A plan of study is presented

The collection and classification of the material of strains is described, and results are discussed supporting the assumption of the relative importance of the S form in pathological conditions

REFERENCES

Alexander H E The Hemophilus group Pp 470 485 in Dubos R J ed Bacterial and mycotic infections of man 3rd ed Pitman Medical Publishing Co Ltd London 1958 820 pp

Alexander H E & Heidelberger M Chemical studies on bacterial agglutination V J Exp Med *71* 1 11 1940

Anderson R A & Schultz O T Immunologic study of strains of Bacillus pfeifferi isolated from a case of meningitis J Exp Med *33* 653 666 1921

Breed R S Murray E G D & Smith N R Bergey's manual of determinative bacteriology 7th ed The Williams & Wilkins Co Baltimore 1957 1094 pp

... *Dingle J H* The pattern of dissociation in Hae... *37* 415 425 1939

...rs in bacterial growth III J Infect Dis *29* 171 ...

Dawson M H Variation in the pneumococcus J Path Bact *39* 323 334 1934

Dawson M H Hobby G L & Olmstead M Variation in the hemolytic streptococci J Infect Dis *62* 138 168 1938

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SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

2. *Examination of the Type Specific Antigens of H. influenzae by means of the Capsular Swelling Method*

By

TOV OMLAND

Received 26 v 69

The capsular swelling or "Quellung" reaction was first described by *Neufeld* (1902). For many purposes this type of antigen antibody reaction is unsurpassed because it enables the investigator to observe the reaction microscopically in a single bacterial cell. Most other methods are time consuming and necessitate the preparation of greater amounts of antigen. For these reasons the method has become valuable in the diagnosis of certain acute infectious diseases. It may thus have been the only practicable method for a rapid typing of pneumococci in the serotherapy of pneumonia used earlier (*Wilson & Miles* 1955, p. 684).

The capsular swelling procedure may be performed in bacterial culture or directly in pathological material (from the patient or after passage through an experimental animal). In general the cultural method provides more controllable conditions but is said to favour the development of R variants, in contrast to the method using pathological material.

As judged from the literature the tendency towards R variation is considerable in H. influenzae under subcultivation (*Tesdal* 1932, *Fothergill & Chandler* 1936, *Chandler, Fothergill & Dingle* 1939, *Tunevall* 1952). Preliminary experiments in the present study were carried out to examine this possibility. It was found that type strains of H. influenzae preserved their type specific property apparently unchanged in ... swellin...
coccus
technic ... u lection i.e. that cultural methods are well suited for the preservation of type spec f ...

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1949

Rosenberg E, Leidy G, Jaffe I & Zamenhof S Studies on type specific substances of Hemophilus influenzae types e and f J Biol Chem *236* 2841 2844 1961
Schafer W & Martin H Über den Wert von H influenzae Befunden bei bakteriologischen Sputum Untersuchungen Z Hyg Infektionskr *146* 77 83 1959
Schaub I C & Foley M K Diagnostic bacteriology 4th ed The C V Mosby Co St Louis 1952 356 pp
Schmidt B, Feldmann S & Dobberstein H Untersuchungen zur Frage der Änderung der biologischen Eigenschaften von Bakterien nach Trocknung und Gefriertrocknung Zbl Bakt (Orig) *172* 573 580 1958
Scott W M The influenza group of bacteria Pp 326-394 in Medical Research Council A system of bacteriology in relation to medicine His Majesty s Stationery Office London 1929 vol II 420 pp
Smith M M Observations on Bacillus (Haemophilus) influenzae with special reference to morphology and colonial characters J Hyg (London) *31* 321–335 1931
Straker E A Serological typing of meningeal strains of H influenzae Lancet 248 p 817 1945
Thjotta T & Avery O T Studies on bacterial nutrition III J Exp Med *34* 455 466 1921
Wilkes Weiss D A comparison of meningeal and other strains of H influenzae J Infect Dis *60* 213 222 1937
Williamson G M & Zinnemann K The occurrence of two distinct capsular antigens in H influenzae type e strains J Path Bact *63* 695 698 1951
Williamson G M & Zinnemann K The degradation of Haemophilus influenzae type e capsular antigens J Path Bact *68* 453–457 1954
Wilson G S & Miles A A Topley and Wilson s principles of bacteriology and immunity 4th ed Edward Arnold Ltd London 1955 vol I 1106 pp
Yabe S Grouping of influenza bacilli Brit J Exp Path 2 197 204 1921
Yagi A (= Iwaka A) Immunisatorische Einteilung der Influenzabazillen Z Immun Forsch *86* 75 79 1935
Zamenhof S & Leidy G Further studies on polyribophosphate and other polysugar phosphates Fed Proc *13* p 327 1954
Zamenhof S, Leidy G, Fitzgerald P, Alexander H E & Chargaff E Polyribophosphate the type specific substance of Hemophilus influenzae type b J Biol Chem *203* 695 704 1953
Zinnemann K Haemophilus influenzae and its pathogenicity Ergebn Mikrobiol Immun Forsch Exp Therap *33* 307–368 1960

It was found that better growth was obtained in the broth (Levinthal broth (*Levinthal* 1918 *Alexander* 1958)) when the tubes were incubated in oblique nearly horizontal position and were shaken occasionally so as to secure a great surface/volume ratio and thereby better aeration An incubation period of 4–6 hours was found most favourable In most cases it secured sufficient growth and good development of capsules—when such were present—at the same time as it was short enough to prevent inhibition phenomena such as might be found when too much precipitable substance had passed into solution One drop of broth culture was mixed with one
mined microscopically usually
illumination The addition of
later omitted as it apparently

The preliminary experiments established that the broth culture method was the most suitable one for the purpose, also because the fluid culture medium was easier to prepare and handle and this method has then been adhered to in the subsequent work

Establishment of Reference Systems of Known Serological Types

The preliminary trials described above had been carried out with the best type systems available at that time *i e* the a and b systems After choosing and adapting the practical procedure for capsular swelling tests however it became necessary to establish reliable standard systems for each one of the known serological types a b c d e and f

Antigens These were prepared from type strains kindly provided by *dr Engbæk* Statens Seruminstitut Kobenhavn The strains were received in freezedried state (date of freezedrying 13 Nov 1951), and shall be referred to in this study under the designations indicated in Table 1

TABLE 1
Designation of Type Reference Strains

Type	Designation
a	a 51
b	b 51
c	c 51
d	d 51
e	e 51
f	f 51

The type reference strains were subcultured once and freez... after ...

... principles described in a previous article (*Omland* 1963)

Antisera Preliminary trials were made with type sera supplied commercially by Burroughs Wellcome England These sera were labelled "agglutinating sera" by the manufacturer and were not used in the subsequent studies

It was planned that all later investigations should be based on the

Tunevall 1952, *Rogers & Zinnemann* 1958), and preliminary experiments had to be carried out to decide which method to adapt in the planned investigations

EXPERIMENTAL

Preliminary Experiments on the Capsular Swelling Method Using Agar Microscopy (Engbæk 1949 Tunevall 1952)

The procedure is based on the employment of Ørskov's agar microscopy The antiserum is applied directly on the agar culture surface so that the capsular swelling may be observed with the organisms in situ Tunevall's modification consists of the pouring of the agar directly on microscopical slides so as to facilitate the observation

Levinthal agar has been used in the following experiments (*Levinthal* 1918 *Alexander* 1958)

A known type system was used to try out the method but it caused difficulties to secure sufficient humidity during the cultivation The arrangement shown in Fig 1 was found satisfactory

The inner Petri dish was thus converted into a moist chamber

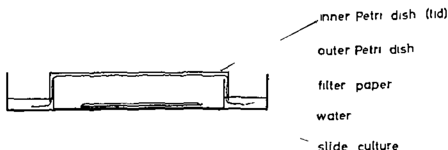


Fig 1
Arrangement for slide culture of H influenzae

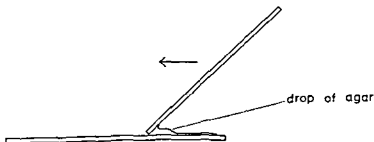


Fig 2
Application of agar on slide (slide culture)

In a series of experiments a modification was tried in which the agar was applied onto the slide in the manner used when preparing blood smears as shown in Fig 2

The very thin agar layer used in the experiments could not be expected to supply enough nutritive material for a longer period of incubation However as it was the question of short incubation (4 6 hours) no problem was met with in this connection The advantage of the modification was that it provided a thin and level layer of agar facilitating focusing and microscopical observation

Preliminary Experiments on Capsular Swelling Methods Using Cultivation in Broth (Alexander 1958 Rogers & Zinnemann 1958)

These methods consist essentially in mixing a drop of broth culture with a drop of antiserum and observing the capsular swelling microscopically on a slide

TABLE 2

Type Reference Systems for Capsular Swelling Tests

Type	Antigen	Antiserum	Capsular swelling titer (serum dilution)
a	a 51	3	1 160
b	b 51	5	1 320
c	c 51	15	1 320
d	d 51	16	1 320
e	e 51	9	1 320
f	f 51	10x	1 80

cutting an ear vein with a razor's blade A technique has also been used which to the author's knowledge, has not been described for such purposes earlier It consists of puncturing one of the ear arteries with a fine needle (gauge 20 or finer) The advantage of this technique is that the blood is obtained under entirely sterile precautions The preliminary tests for potency have been carried out with the capsular swelling method, and later during the study by means of agar gel precipitation

The type systems indicated in Table 2, have been selected as reference systems for the examination of the material of strains

These systems were set up after cross-matching type antigens and type antisera in all possible combinations Out of several sera for each type, the above listed have been chosen, because they have given the most distinct results Capsular swelling titres are shown in Table 2

In these systems the capsular swelling reactions were easily observed in the homologous combinations, whereas no such reactions were seen in heterologous combinations However, inherent difficulties are involved in the observation of capsular swelling systems in general In any particulate system in fluid halo effects will complicate the picture In cases presenting great capsular dimensions, these optical effects do not confuse observation, but in cases of moderate or thin capsules it may be open to discussion whether peripheral optical phenomena are due to capsular swelling or to such unspecific effects

RESULTS

Application of the Capsular Swelling Method on the Material of Strains

Table 3 shows the distribution of the material of collected H influenzae strains described in a previous paper (Omland 1963) according to origin of the pathological material and serotype, as determined by capsular swelling tests

Figures in brackets represent equivocal results, and it is seen from the table that the numbers of such results constitute more than one fourth of the total number of typable strains

Tables 4 and 5 show the distribution of types by category of variation, in pure, respectively mixed, culture

use of hyperimmune rabbit sera, and in the following a detailed description shall be given of the preparation of such sera

Albino rabbits weighing about 2 kg were selected for inoculation Care was taken that the antigen to be used had undergone as little denaturation as possible Consequently it was decided to use live organisms in the logarithmic phase of growth As it was decisive to preserve the capsular antigen, which is shown to be in optimal condition only in very young cultures (*Engbæk* 1949), it seemed particularly important to adhere to such principles The following practical procedure was adopted. Freezedried organisms were seeded directly, or after one subculture on blood agar, onto a plate of chocolate agar (heated blood agar) and incubated for 6 hours at 37° C in a moist chamber A Gram-stained smear was examined as a control of contaminants If found satisfactory, the growth was harvested in about 2 ml of sterile physiological saline and a measured volume was injected without further treatment into an ear vein of the animal During the first part of the study the opacity of the suspension was measured by means of a colorimeter (model B Lange), and adjusted to the equivalent of 1000 millions staphylococci per ml (*i e* about 3000 millions H influenzae per ml) (*Cunningham & Timothy* 1924, cit *Mackie & McCartney* 1953, p 275) During the later stages of the work this adjustment was omitted, without appreciable effect on the results The volumes injected have varied from 0 05 to 1 0 ml, however most frequently starting at 0 10 ml and finishing at 0 50 ml, with gradual increase during the series The intervals between the inoculations have ranged from 2 to 6 days, except for occasional longer pauses The animals have received from 10 to 25 inoculations (average about 15), *i e* from about 3 ml to about 7 5 ml antigen suspension The bleeding (heart puncture) has ordinarily been started after 8 to 10 injections During the period of bleeding the animals have received one injection weekly, and the bleeding has been performed 4–5 days after the injection A weight record has been kept for each animal, and the inoculation schedule has been interrupted in case of weight decrease, suggesting that the immunization has not been well tolerated Only very few cases of serious complications have occurred, and the most obvious pathological feature has been paraparesis of the hind legs as described by *Tesdal* (1932)

The serum portions have been inactivated at 56° C for 20 30 minutes, and all satisfactory portions from each animal have been mixed into a serum pool, and labelled with the code number of the respective animal The sera have been kept in frozen state (—20° C) in small portions, so as to avoid repeated freezing and thawing during the work In most instances a preservative (Merthiolate "Lilly") has been added to the serum pools before storing

During the immunization preliminary tests for potency of the sera have been carried out For this purpose only small amounts were needed, and have been obtained partly by the conventional method of

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Origin	Serological type a	b	c	d	e	f	Total no. typable	Total no. of strains	Per cent typable*
Nose	3 (2)	8 (3)	0 (1)	2 (0)	3 (1)	4 (0)	20 (7)	95	21
Throat	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3	0
Sputum	0 (0)	0 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (1)	7	0
Bronchus	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	1 (0)	5	20
Sinus	1 (0)	0 (1)	0 (0)	0 (0)	1 (0)	1 (0)	3 (1)	24	12
Ear	0 (0)	2 (0)	0 (0)	0 (1)	1 (0)	0 (0)	3 (1)	10	30
Conjunctiva	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2	0
Vagina	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1	0
Cerebrosp. fl.	0 (0)	7 (4)	0 (0)	0 (0)	1 (0)	0 (0)	8 (4)	14	57
Blood	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	2	50
Totals	4 (2)	18 (9)	0 (1)	3 (1)	6 (1)	5 (0)	36 (14)	163	22

Figures in brackets: equivocal results

* Calculated from the unequivocal results

TABLE 4

Distribution of H. influenzae Strains Isolated in PURE Culture According to Category of Variation and Serotype as Determined by Capsular Swelling Tests

Morphology	Serological type a	b	c	d	e	f	Total no. typable	Untypable	Totals
Mucoid	1 (1)	12 (4)	0 (0)	0 (1)	2 (0)	1 (0)	16 (6)	3	25
Smooth			0 (1)				0 (1)	33	34
Rough									0
Totals							16 (7)	36	59

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TABLE 5

Distribution of H. influenzae Strains Isolated in MIXED Culture According to Category of Variation and Serotype as Determined by Capsular Swelling Tests

Morphology	Serological type a	b	c	d	e	f	Total no. typable	Untypable	Totals
Mucoid	3 (1)	6 (3)	0 (0)	3 (0)	4 (1)	4 (0)	20 (5)	10	35
Smooth		0 (2)					0 (2)	66	68
Rough								1	1
Totals							20 (7)	77	104

Figures in brackets: equivocal results

It is seen that a total of 13 M-strains are untypable by capsular swelling tests, while on the other hand 3 S-strains are typable (doubtful type) Attention is called to the finding that as many as 33 untypable S strains have been isolated in pure culture Thirty of these strains were from the upper part of the respiratory tract and adnexa (nose, sinus, and ear) This observation is an extension of the findings described in a previous article (*Omland* 1963), underlining the registered preponderance of S forms

DISCUSSION

Among 163 H influenzae strains 36 were typable by capsular swelling tests (22 per cent) In addition capsular swelling reactions were registered in 14 of the strains, although the type was uncertain

The most common type was type b The other types occurred by decreasing frequency as follows e, f, a, d No definite type c strains was encountered The fact that the interpretation of the capsular swelling reaction was doubtful in 14 out of 50 strains, is remarkable, and may be a reflection of certain methodological disadvanteges involved in the capsular swelling technique

The findings presented in Table 4 and 5, particularly as concerns the S forms, agree with the statement made in a previous article on the importance of such forms, even though they are ordinarily untypable in Pittman's type system (*Omland* 1963)

SUMMARY

Some general considerations are presented on the capsular swelling ("Quellung") technique The adaptation of this technique to the study of H influenzae is described Finally the results of the examination of the material of strains with such technique, are presented

REFERENCES

Alexander H F The Hemophilus group Pp 470–485 in Dubos R J ed Bacterial and mycotic infections of man 3rd ed Pitman Medical Publishing Co, Ltd London 1958 820 pp

Chandler [illegible] dissociation in Hae- [illegible]

Engbæ [illegible] ktionens forløb hos [illegible] – A Busck, København 1949 245 pp

Fothergill I D & Chandler C A Observations on the dissociation of meningitis strains of H Influenzae J Immunol *31* 401 415 1936

Leventhal W Bakteriologische und serologische Influenzastudien Z Hyg Infektionskr *86* 1 24 1918

Mackie T J & McCartney [illegible] Livingston [illegible]

Neufeld F [illegible] Agglutina [illegible]

Omland T Serological studies on Haemophilus influenzae and related species I Acta path et microbiol scandinav 57 268–78, 1963

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Bronchus	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	1 (0)	5	20
Sinus	1 (0)	0 (1)	0 (0)	0 (0)	1 (0)	1 (0)	3 (1)	24	12
Ear	0 (0)	2 (0)	0 (0)	0 (1)	1 (0)	0 (0)	3 (1)	10	30
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[illegible] 1949 245 pp

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MUNICIPAL VIROLOGICAL LABORATORY AND DEPARTMENTS OF PATHOLOGY AND PEDIATRICS UNIVERSITY OF GOTHENBURG SWEDEN

A CASE OF INTRAUTERINE VACCINIA

By

ERIK LYCKE, CHRISTER AHRÉN, RUNE STENBORG, GUNNAR BERNLER and SVEN SPETZ

Received 1 viii 62

In spite of frequent vaccinations with vaccinia virus of pregnant women the reported incidence of congenital vaccinia is very low (1–5) Indeed *Bellows et al* (3) stated that 'smallpox vaccination during pregnancy does not increase the incidence of congenital malformations, still births, abortions or of infant deaths

However, vaccination of pregnant women has undoubtedly caused a few cases of injury to the foetus *Jenner* (6) described how, 5 weeks after the vaccination, a woman was delivered of a still born foetus with several pustules on erythematous bases Seven other cases have been cited by *Lynch* (1) in which abortions or still births presumably were caused by vaccination during pregnancy Lynch observed a child who died shortly after birth and had generalized pustular eruption, pulmonar atelectasis and necroses in the liver The mother had been vaccinated about one month earlier In a case reported by *MacDonald & MacArthur* (7) the mother was vaccinated with a severe primary reaction three months before delivery Foetal vaccinia was diagnosed from the clinical and pathological features, including nuclear inclusion bodies Two cases have been observed in Holland (8 9) *Wielenga et al* (9) described an intrauterine infection in a 27½ weeks old foetus with virus isolated from the skin and placenta This seems to be the first case where the diagnosis was virologically confirmed The mother was unvaccinated Her first child however, had been vaccinated 5 weeks before delivery of the infected foetus

CASE REPORT

[illegible]

On March 17 about 5 weeks after the vaccinat[illegible]

girl [illegible]

mote [illegible]

the [illegible]

blee [illegible]

The [illegible]

Rogers K B & Zinnemann K The recognition of non capsulated strains of Haemophilus influenzae in unusual sites P 324 in Tunevall G ed Abstr VIIth Int Congr f Microbiol Almqvist & Wiksell Uppsala 1958

Tesdal M Experimental encephalo myelopathy in rabbits following intravenous injection of a smooth form of Bacillus influenzae Pfeiffer recovered from a patient with meningitis Acta path et microbiol scandinav Suppl *11* 191 200 1932

Tunevall G Studies on Haemophilus influenzae Type characteristics Acta path et microbiol scandinav *30* 203 212 1952

Wilson G S & Miles A A Topley and Wilson s principles of bacteriology and immunity 4th ed , Edward Arnold Ltd London 1955 vol I 1106 pp



Figs 3 and 4

Fig 3 Preparation of skin sample from one of the lesions of the soles Van Gieson s stain Magnification 50 ×

Fig 4 Preparat on of placenta sample Van Cieson s stain Magn f cation 50 ×



Figs. 1 and 2.
The lesions in the face and on the soles The pictures were taken when the child was two days old

The ch ld was placed in couveuse with oxygen supply However per ods of apnoea gradually became longer and the skin lesions necrotized especially in the face and on the feet (F gs 1 and 2) The ch ld died on the e ghth day

Pathological Findings

At autopsy the child was found to have generalized skin lesions in the form of 0 5 to 1 cm crusted efflorescences The lesions are particularly abundant in the face and on the back arms and legs On the soles right hand and ridge of the nose they have turned into open necrotic ulcerations The lungs and liver contain scattered pinhead sized yellowish white foci with a dry cut surface and firm consistency In the left lung similar foci are confluent to a large infiltration extending out to the pleural surface which is fibrin coated and adheres to the thoracic wall The liver in addition contains extensive subcapsular necroses The mesenteric lymph nodes are enlarged but not necrotic and contain no focal lesions The central nervous system other parts of the respiratory and digestive organs the urogenital organs and the endocrine organs are grossly normal No malformations are present

The placenta is of normal size considering the duration of pregnancy and has grossly normal structure but contains scattered yellowish white ricegrain sized foci which are diffusely demarcated and of soft consistency Histologically the skin lesions (Fig 3) appear like large epidermal vesicles filled with mononuclear cells polymorphonuclear leukocytes and detritus Towards the centre these processes become ulcerated The surface is covered with a crust The cutis and surrounding epidermis are oedematous and invaded by inflammatory cells eosinophilic leukocytes are present in abundance The foci in the parenchymatous organs are of uniform appearance and consist of diffusely demarcated disseminated necroses with remarkably weak inflammatory cell reaction in the neighbourhood The infiltrations are predominantly composed of mononuclear cells and eosinophilic leukocytes Near some of the placental foci (Fig 4) there are abundant calcified deposits The cord and umbilical membranes present no conclusive inflammatory changes Inclusion bodies are present in stroma cells around placental necroses and also in degenerating epidermal cells around the foetal skin lesions (Figs 5 and 6) The inclusions are round or oval usually surrounded by a distinct halo They occur solely in the cytoplasm and their strongly positive response to Lendrum s *Phloxin Tartrazin and* Feulgen s staining procedures suggest that they contain DNA The cell nuclei exhibit degenerative changes and marginal chromatin clumping No intranuclear inclusion bodies are visible

Virology

Samples of the placenta were examined virologically The homogenized tissue was inoculated as a 10 per cent suspension on chorioallantoic membranes of twelve days old chick embryos and into HeLa



Figs 5 and 6

Fig 5 Cytoplasmic inclusion bodies in degenerating epidermal cells Preparation of skin Feulgen's stain Magnification 400 ×

Fig 6 Cytoplasmic inclusion bodies in cells of placenta preparation Feulgen's stain Magnification 400 ×

cell cultures After three days of incubation at 36 5° C, plaques, morphologically identical with vaccinia virus pocks, were demonstrable on the egg membranes and a cytopathogenic agent typed as vaccinia virus was isolated in the cell cultures

Preparations of placenta and skin were also tested by means of fluorescent antibody technique (10) As the available samples had been used for the histopathological examinations and were formalin fixed, they were deformalinized prior to the fluorescent antibody examination Two μ sections were used for conjugation with serum The antivaccinia serum was prepared by hyperimmunization of rabbit Labelling with fluorescein isothiocyanate and fractionation of labelled serum on DEAE column was performed according to the methods of Riggs et al (11) Placental and skin sections exhibited a yellowish-green fluorescence at the central parts of the necrotic lesions after testing with antivaccinia rabbit serum (Figs 7 and 8) but not after testing with serum taken prior to immunization of the rabbit

SUMMARY AND COMMENTS

A woman of 29 was vaccinated in the 23rd week of pregnancy Five weeks later she bore an immature girl with severe skin lesions, who died on the eighth day Vaccinia virus was isolated from placenta and vaccinia antigen was demonstrated in placenta and skin samples by fluorescent antibody technique Cytoplasmic inclusion bodies positive to Feulgen's stain were demonstrated in epidermal and stroma cells

The described pathological

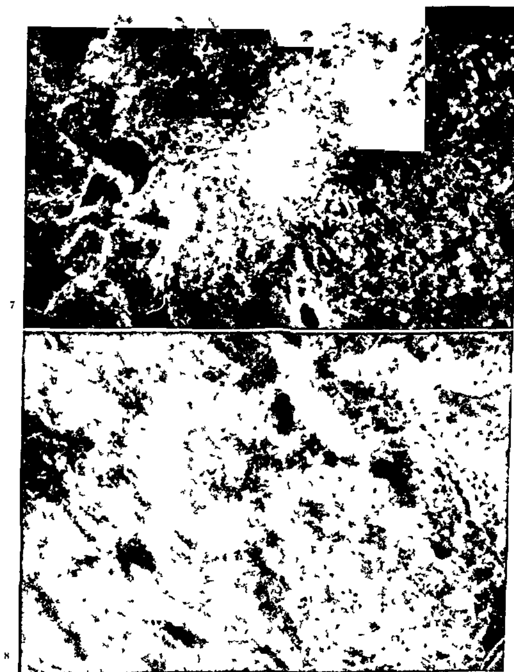
enga et al, and the present

thereto

After vaccination the mother here discussed developed a local primary reaction with a slight rise in temperature as the only general sign of infection Virus transmission to the foetus was not concomitant to a severe vaccination reaction in the mother nor did the mother develop any signs of infection during the rest of the pregnancy A normal type of reaction in the pregnant woman thus does not exclude the possibility of such a severe vaccination complication as intrauterine vaccinia in the child In fact the case reported by *Wielenga et al* shows that mere contact by a non vaccinated pregnant woman with a newly vaccinated individual during the period of local reaction may be hazardous to the foetus

Involvement of placenta and internal organs indicates a haematogenic virus transmission Whether transmission of virus may also occur by the amniotic fluid remains to be proved

Intrauterine vaccinia infection is a rare complication to vaccination As mentioned by Wielenga et al, it has been assumed that vaccination can be performed after the first trimester without risk to the foetus However the intrauterine infection described, as well as the one re-



Figs 7 and 8

Fluorescent areas in preparations of skin (Fig. 7) and placenta (Fig. 8). The preparations were treated with rabbit antivaccinia serum labelled with fluorescein thioisocyanate. Magnification 130 ×

FROM DEPARTMENT OF MICROBIOLOGY AND HYGIENE, THE VETERINARY COLLEGE OF NORWAY, OSLO (HEAD: PROFESSOR STEINAR HALGE)

PHOSPHOLIPASE D ACTIVITY IN A NON HAEMOLYTIC CORYNEFORM BACTERIUM

By

KÅRE FOSSUM and TORE HOYEM

Received 19 ix 62

The formation of lecithinases (phospholipases) by microorganisms has been extensively studied in recent years in an attempt to use it in the taxonomy of bacteria and to find a connection with toxicity and virulence

According to the mode of splitting lecithin, the lecithinases may be divided into four groups (1)

Enzyme	*Substrate*	*Products*
Phospholipase A	Lecithin	
Phospholipase B	Lysolecithin	acid
Phospholipase C	Lecithin	
Phospholipase D	Lecithin	

Lecithinase production is reported for both Gram positive and Gram negative bacteria

Esselmann & Liu (2) and *Bottcher* (3) have reviewed the work done in this field According to these authors the following Gram positive bacteria are lecithinase producers *Clostridium perfringens, Clostridium novyi Clostridium bifermentans, Clostridium haemolyticum, Bacillus cereus Bacillus mycoides, Bacillus anthracis*

Several types of acid fast bacteria are also reported to be lecithinase producers (2)

Esselmann & Liu who studied the lecithinase production by Gram negative bacteria found several species within the family *Pseudomonadacea* and the genus *Serratia* to be lecithinase producers *Vibrio comma* and *Vibrio El Tor* together with an other vibrio species were also found to produce lecithinase All active lecithinase producers in their study (2) were found to be haemolytic The Gram positive lecithinase producers mentioned above are also haemolytic together with other microorganisms which are shown to be lecithinase producers, such as *Actinomyces albus* (*Streptomyces albus*) and *Actinomyces coelicolor* (*Streptomyces coelicolor*) (3 4)

The purpose of the present work was to study the lecithinase activity in a nonhaemolytic coryneform bacterium earlier described by *Hagen & Fossum* (5)

ported by *Wielenga et al*, shows that infection of the foetus also may occur in the second and third trimesters Therefore it might be advisable to avoid vaccination of pregnant women

REFERENCES

1 *Lynch F* Dermatologic conditions of the foetus with particular reference to variola and vaccinia Arch Derm Syph *26* 997 1932
2 *Greenberg M A Yankauer S Krugmann J J Osborn R S Ward & Dinces J* Effect of smallpox vaccination during pregnancy on incidence of congenital malformations Pediatrics *3* 456 1949
3 *Bellows Majorie T Hyman Mary E & Merrit Katherine K* Effect of smallpox vaccination on the outcome of pregnancy Public Health Reports *64* 319 1949
4 *Mac Arthur P* Congenital vaccinia and vaccinia gravidarum Lancet *263* 1104 1952
5 *Abramowitz* Vaccination and virus diseases during pregnancy South Afric Med J *31* 1 1957
6 *Jenner E* Two cases of smallpox infection communicated to the foetus in utero under peculiar circumstances Med Chir Tr London *1* 271 1809
7 *MacDonald A M & MacArthur P* Foetal vaccinia Arch Dis Child *28* 311 1953
8 *Wiersum A K* Variolois bij een foetus Ned Tijdschr Geneesk *100* 971 1956
9 *Wielenga G van Tongeren H A E Ferguson A H & van Rijssel Th G* Prenatal infection with vaccinia virus Lancet *1* 258 1961
10 *Coons A H & Kaplan M H* Localization of antigen in tissue cells II Improvements in an method for the detection of antigen by means of fluorescent antibody J Exp Med *91* 1 1950
11 *Riggs J L Loh P C & Eveland W C* A simple fraction method for the preparation of fluorescein labelled gamma globulin Proc Soc Exp Biol Med *105* 665 1961

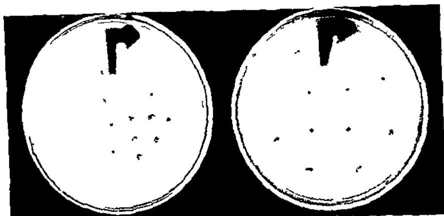


Fig 1 Fig 2

Fig 1 Two different strains of *Corynebacterium pyogenes* (quadrant I and III clockwise from the arrow) and two different strains of the nonhaemolytic corynebacterium bacterium (quadrant II and IV) grown on egg yolk agar for two days in a 10 per cent CO atmosphere at 37° C.

Fig 2 Three colonies of each of the following strains (clockwise from the arrow) *Bacillus cereus* *Staphylococcus aureus* *Serratia marcescens* and *Bordetella bronchiseptica* grown on egg yolk agar for 24 hrs at 37° C



Fig 3

The nonhaemolytic bacterium cultured in egg yolk broth Reading from left to right the tubes are incubated for 6 5 4 3 and 2 days The tube to the right is a control containing uninoculated medium

also distinct after one day of incubation *Corynebacterium pyogenes* gave no such zone

Some other bacteria gave a similar zone when grown on egg yolk agar In Figure 2 are shown colonies of *Bacillus cereus, Staphylococcus aureus* *Serratia marcescens* and *Bordetella bronchiseptica* Around the colonies of *Bacillus cereus* an opaque zone developed, extending about

MATERIALS AND METHODS

Strains of bacteria used.—The organisms used in the present experiments were identical to those used in the work reported by *Hagen & Fossum* (5) and are referred to as non haemolytic coryneform bacteria. Comparative studies were also carried out with different strains of *Corynebacterium pyogenes* that had been isolated from various pathological processes in various species of domestic animals. Other bacteria as *Bacillus cereus*, *Serratia marcescens* and *Staphylococcus aureus* were used for comparison. Furthermore *Bordetella bronchiseptica* was included as a negative control. For the demonstration of lecithinase activity by paper chromatography only three strains of the nonhaemolytic coryneform bacterium and one strain of each species of *Corynebacterium pyogenes*, *Bacillus cereus* and *Bordetella bronchiseptica* were used.

Cultivation of the bacteria.—The nonhaemolytic coryneform bacterium and *Corynebacterium pyogenes* were cultured in an atmosphere containing 10 per cent CO. The bacterial growth and the enzymatic activities were stimulated under this particular incubation condition for both *Corynebacterium pyogenes* (6, 7) and for the nonhaemolytic coryneform bacterium (5). The other bacteria were incubated in ordinary atmosphere and at the optimal temperature for each strain. The bacteria were cultured on egg yolk agar (equal parts of 3 per cent nutrient agar and a 10 per cent egg yolk suspension in nutrient broth).

Furthermore the bacteria were cultured in egg yolk broth. The optimal incubation time for lecithinase production varied for the various organisms used.

The nonhaemolytic coryneform bacterium and *Corynebacterium pyogenes* subjected to paper chromatography were cultured in egg yolk broth for seven days. *Bacillus cereus* was incubated for 24 hrs in the same medium.

Choline determination.—Choline determination was performed with the Florence reagent according to the method used by *Esselmann & Liu* (2) and *Klinge* (8).

Paper chromatography.—A paper chromatographic method was worked out to serve as a supplement to the determination of choline by the Florence reagent.

100 ml of broth cultures cultured as already described were centrifuged at 10 000 × g. Fatty material was separated at the top of the supernatant and was removed. The remaining clear supernatant was shaken with an equal amount of chloroform. After centrifuging and further removal of the chloroform phase the aqueous phase was shaken several times with equal volumes of ethyl ether.

The lipid free aqueous phase was then mixed with an equal amount of ice cold 1.2 M perchloric acid in order to precipitate proteins. After one hour at 0° C the mixture was centrifuged. The supernatant was adjusted to pH 7 at 0° C whereby the perchloric acid was precipitated as the potassium salt. The supernatant from this precipitation was evaporated to dryness on a boiling water bath or freeze dried and residue dissolved in 0.5 ml of 96 per cent ethanol. After centrifugation the supernatant was used for chromatography.

25–100 µl aliquots were subjected to descending paper chromatography on Whatman No. 1 filter paper. Three different solvents were used: (a) n butanol acetic acid water (2:1:1), (b) n butanol HCl (98:2) saturated with water and (c) isopropanol HCl H_2O (70:15:15).

For the detection of the spots the chromatograms were sprayed with 1 per cent potassium ferrocyanide followed by 1 per cent cobalt chloride (9).

RESULTS

In Figure 1 are shown the zones appearing around the colonies of the nonhaemolytic coryneform bacterium when grown on egg yolk agar. A sharply demarcated zone of lysis developed around the colonies. Close to the colonies there might be an opaque zone. In reflected light the zones looked "oily", rainbow coloured. The zones from different strains varied in size. After two days of incubation under the particular conditions established the diameter varied from 4 to 7 mm. The zone was

TABLE 1

Rf Values Obtained by Paper Chromatography of Choline from Various Sources

Organism or reference	Solvent a	Solvent b	Solvent c
[illegible]	0 63	0 08	0 58
[illegible]	0 63	0 09	0 58
[illegible]	0 63	0 08	0 59
[illegible]	–	–	–
[illegible]	–		–
[illegible]	–	–	–
[illegible]	0 63	0 08	0 66
Control (sterile incubated medium)	–	–	–

Composition of solvents consult text

DISCUSSION

All the bacteria previously described as lecithinase producers also possess haemolytic activity This had led to the hypotheses that there might exist a connection between these two activities

We have observed a lecithinase positive bacterium which is nonhaemolytic This bacterium greatly resembles *Corynebacterium pyogenes* and are found in the same pathologic processes (5)

In this work we have only searched for phospholipase D activity We found that every strain of the nonhaemolytic coryneform bacterium investigated liberated choline when grown in egg yolk broth

In the case of *Streptococcus aureus, Gillespie & Alder* (11) found that the egg yolk reaction seemed to be associated with the lipase activity

In one of the chromatography solvents (c) there is a slight difference between the Rf value of choline, and that of the bacterial choline reacting substances It is not known whether this is due to the chromatography technique, or if it is significant This point is, however, under consideration

SUMMARY

The ability of producing choline from lecithin has been demonstrated in strains of a nonhaemolytic coryneform bacterium This shows that lecithinase D production does not necessarily be combined with haemolytic activity

Even though the properties of the nonhaemolytic coryneform bacterium in many respects resemble those of *Corynebacterium pyogenes*, the lecithinase D activity of the former makes a sharp distinction between them

In addition to the Florence reagent test, a *paper chromatography* method was developed for the demonstration of choline

1 cm from the colonies Some strains of *Staphylococcus aureus* developed a zone of lysis similar to the zone around the colonies of the non-haemolytic coryneform bacterium Around the colonies of *Serratia marcescens* a similar zone developed which is due to a phospholipase (10) *Bordetella bronchiseptica* gave no zone

When cultured in egg yolk broth the non-haemolytic coryneform bacterium lead to the development of a turbidity which after some time gathered at the top, leaving a clear layer beneath In Figure 3 is shown the development of this opacity and arising of the curd Already after two days of incubation a turbidity was seen, while the curd arose after this lapse of time After several days of incubation the layer beneath the curd became clear *Bacillus cereus* and *Serrati marcescens* gave the same reaction, while no such reaction could be seen with *Corynebacterium pyogenes* and *Bordetella bronchiseptica* *Staphylococcus aureus* might give a slight opacity, but the curd does not rise to the top in this case

According to the results obtained with the Florence reagent and with paper chromatography, the nonhaemolytic coryneform bacterium produces choline when cultured on egg yolk medium *Bordetella bronchiseptica* and *Corynebacterium pyogenes* gave no choline reaction with the method used

Figure 4 shows the choline-periodide crystals obtained with supernatant from the nonhaemolytic coryneform bacterium culture

Table 1 gives the Rf values obtained with the three solvent systems used in paper chromatography



Fig 4
Choline periodide crystals obtained with the culture supernatant of the nonhaemolytic coryneform bacterium

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NON SPECIFIC STREPTOLYSIN O INHIBITION IN DISEASES OF THE LIVER AND BILIARY SYSTEM

By

J HALLÉN

Received 23 ix 62

During the last 3 decades an increased antistreptolysin O titre (AST) has been regarded as a contributory sign of streptococcal infection It was however soon realised that high AST titres sometimes were due to serum components other than antibodies to streptolysin In such cases the reaction is said to be non specific

Non specific reactions have been reported e g in liver diseases of which viral hepatitis has received extensive attention in nephrosis and tuberculous pleurisy (*Sievers* 1947 *Packalen* 1948 *Kalbak* 1947 *Westergren* 1948) Such reactions have often been ascribed to an abnormally high concentration of various serum lipoproteins (*Badin & Laban* 1959 *Hewitt & Todd* 1939 *Scheiffert et al* 1957 1959 *Stollerman* 1954) Various methods have been used to separate the lipids from the serum to demonstrate an aetiological correlation if any between the lipoids and AST as well as to decide whether or not an increase of the titre is specific But the procedures hitherto described are either too complicated for routine use (*Packalen* 1948) or have proved unsuitable for some other reason (*Oker Blom et al* 1950)

For several years we have studied sera from patients with hypergammaglobulinaemia routinely for a number of serological reactions (*Wallenstr m & Winblad* 1958) Those examinations revealed among other things that of 103 sera from patients with liver cirrhosis the AST was definitely increased in 49 and slightly increased in 17 No correlation was found between the AST and the serum γ globulin concentration but in patients with a serum bilirubin concentration exceeding 3 mg/100 ml the AST was often increased (Fig 1) In an attempt to find out the cause of this high frequency of increased AST in cirrhosis the β lipoproteins in patients with jaundice and more or less severe biliary obstruction were studied for effects if any on the AST

REFERENCES

1 *Hayaishi O* Phospholipases Methods in Enzymology I p 660–674 New York 1955
2 *Esselmann M T & Liu P V* Lecithinase production by Gram negative bacteria J Bacteriol *81* 939 945 1961
3 *Bottcher K* Lecithinase activity of some sporogenous soil bacteria Microbiologiya *30* 673 678 1961
4 Bergey's Manual of Determinative Bacteriology Seventh Edition p 753 and 756 Baltimore 1957
5 *Hagen O & Fossum K* A nonhemolytic coryneform diphtheroid bacterium isolated from pyogenic processes in domestic animals Nord Vet Med *14* 407 414 1962
6 *Jepsen Aa* Diagnostisk bakteriologi og levnedsmiddelbakteriologi p 62 København *1960*
7 *Hartwig H & Grund S* Atypische Corynebakterien beim Rind Zbl Bakt Orig *179* 499 508 1960
8 *Klinge K* Differenzierung der im Wasser vorkommenden Bakterien des Genus Pseudomonas durch die Eigelbreaktion Arch Hyg *141* 348 360 1957
9 *Dawson R M C Elliott D C Elliott W H & Jones K M* Data for Biochemical Research London 1959
10 *Klinge K* Die Eigelbreaktion und die Wirkung von Mikroorganismen auf den Eigelbkomplex Arch Hyg *141* 334 347 1957
11 *Gillespie W A & Alder V G* Production of opacity in egg yolk media by coagulase positive Staphylococci J Path Bact *64* 187 200 1952

RESULTS

The AST was high or very high in 12 jaundiced patients with biliary stasis due to gallstones in 9, and to carcinoma in 3 (Table 1) One of the patients with gallstone was examined on 2 occasions during regression of his jaundice and the decrease of the icterus index was found to be accompanied by a decrease of the AST – In 5 patients with hepatitis the AST varied In the patient with the lowest icterus index the titre was normal – In 1 patient with biliary cirrhosis the titre equalled the highest noted in patients with extrahepatic obstruction – No close correlation was found between the severity of bilirubinaemia and the AST

TABLE 1

AST in 18 Cases of Jaundice of Varying Origin

Diagnosis	Bil s mg 100 ml	AST units	Diagnosis	Bil s mg 100 ml	AST units
Biliary obstruction			Biliary obstruction		
stone	17 5	56000	cancer	28 0	512000
	17 0	2000	"	14 5	7000
	16 0	5000		11 5	2000
	11 5	7000	Hepatitis	15 0	1600
*	7 8	512000	"	7 5	6000
	5 1	720	"	4 4	360
*	4 8	3500	"	4 1	425
	4 0	6000	"	2 7	110
"	3 9	850	Biliary cirrhosis	16 0	256000

* Same patient

TABLE 2

AST before and after Precipitation of β Lipoproteins in Dieases of the Liver and Biliary System

Diagnosis	AST before	AST after
	precipitation of β lipoproteins	
Biliary obstruction stone	7000	55
"	2000	97
	720	95
" cancer	7000	80
Hepatitis	2000	<35
"	6000	110
Fatty liver + obstruction	1600	35
Hepatitis + obstruction	1500	35
Cardiac failure	8000	250
Biliary cirrhosis yellow serum	425	65
Cirrhosis yellow serum	256000	125
Cirrhosis "normal" serum	425	110
	12000	95
	6000	300
" "	600	210

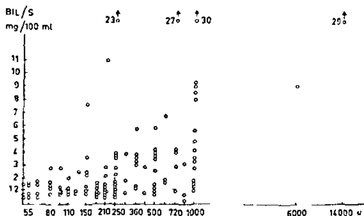


Fig 1

The accumulation of AST values of 1000 units is due to the fact that titration was formerly discontinued at that level

MATERIAL

The sera used to demonstrate any correlation between the AST and cholestasis originated from a number of patients with jaundice of varying severity and origin

The sera used for the β lipoprotein precipitation described later originated from (a) 10 patients with a serum bilirubin level of more than 4 mg/100 ml selected at random the diagnoses being unknown, from the archives of the Department of Clinical Chemistry, (b) 5 patients with liver cirrhosis including 2 with markedly yellow serum and 3 with serum of normal gross appearance but with known increased AST-titre, (c) 7 patients with diseases presumably secondary to streptococcal infections—3 with rheumatic fever, 3 with acute tonsillitis (growth of β haemolytic streptococci on culture of throat swabs in 2 cases studied) and 1 with acute nephritis

METHODS

Antistreptolysin reaction—Streptolysin from a broth culture of the S 84 strain was used The broth for streptolysin production was made on ox heart with 2 per cent peptone 0 2 per cent glucose 0 2 per cent $NAHCO_3$ and 0 1 per cent Na_2HPO_4 pH being adjusted to 8 0 The streptolysin broth was centrifuged and filtered through a Seitz filter The amount of streptolysin units was determined by titration against standard antistreptolysin serum after reduction by a 0 4 per cent sodium pyrosulphite solution The standard antistreptolysin globulin was obtained from the Danish State Serum Institute of Copenhagen A suitable amount of streptolysin broth containing 1 unit/ml was reduced by a 0 4 per cent sodium pyrosulphite solution The patient s serum was diluted with normal saline in small tubes to a total volume of 1 ml per tube and was inactivated 0 5 ml of streptolysin was added to each tube After incubation of this mixture in a 37° C water bath for 15 minutes 0 5 ml of a 5 per cent suspension of sheep erythrocytes was added The mixture was then incubated at 37° C for 30 minutes and allowed to stand for 1 hour or overnight at 4° C before the amount of haemolysis was determined colorimetrically The amount of antistreptolysin units present was derived from the number of streptolysin units inactivated

β-lipoprotein precipitation—The β lipoproteins were precipitated by the method of *Burstein & Samaille* (1958) with addition of 0 02 ml of 10 per cent dextran sulphate[1] and 0 1 ml of 1M calcium chloride solution to 1 ml of serum One hour later the mixture was centrifuged for 10 minutes at 1500 G The clear supernatant was decanted and its AST was determined

1 Sodium dextran sulphate (from dextran with M W 5 × 10^5) from Pharmacia Uppsala, Sweden

In diseases secondary to streptococcal infection such precipitation was followed only by a moderate depression of the titre which never even approached normal An increased concentration of the β lipoproteins thus appears to cause inhibition of streptolysin which must be regarded as non specific

The method used can thus distinguish between a non specific increase of the AST due to an increase in the concentration of the β lipoproteins and an elevation due to streptococci The method is simple and lends itself well to routine use

In about one fourth of the patients with liver cirrhosis and an increased AST the serum bilirubin level was normal or only slightly elevated (Fig 1) Unfortunately these patients had been examined before the use of the above mentioned method at our laboratory Three patients with cirrhosis and with apparently normal serum and high AST were studied however and a non specific increase of the AST was noted in all of them Consequently the titre should be checked after precipitation of the β lipoproteins in all patients with a high AST whether or not the serum is yellow

The minimum difference in titre necessary to regard an increased AST as non specific requires investigation on a larger series

Analysis by this method of the AST increase in different types of hyperlipaemia as well as in rheumatoid arthritis and tuberculous pleurisy with high titres is desirable This also holds for other conditions with increased AST not related to streptococci This would help to reveal whether factors other than the β lipoproteins can act as non specific streptolysin inhibitors

It is worth mentioning that a 74 year old woman presenting carcinoma of the colon and liver cirrhosis but no signs of biliary obstruction and streptococcal infection presented AST — 20000 units On serum electrophoresis an abnormal globulin of myeloma type (M component) was seen This component was isolated and found to possess almost all of the streptolysin inhibiting property of the serum Other streptococcal antibodies could not be demonstrated Another woman—65 years old and healthy except for an acute bronchitis in 1960—had an M component in her serum and AST — 80000 units The abnormal globulin has not been isolated yet but it seems probable that the M component is the cause of the AST elevation also in this case At postmortem of the former case no signs of myeloma were found nor has this diagnosis been proved clinically in the latter As expected the AST fall in both was only moderate on β lipoprotein precipitation

SUMMARY

In liver diseases especially with associated biliary obstruction the AST is often increased This is regarded as a non specific sign The increase of the titre is due mainly to the increased concentration of the

The AST (normal. < 210–250 units) was determined before and after precipitation of the serum β-lipoproteins in different diseases of the liver and biliary tract (Table 2) In 5 cases of extrahepatic cholestasis a distinctly elevated AST fell to values clearly below 210 units A similar reduction was noted for 2 patients with hepatitis – Two patients with clinical signs of biliary stasis showed no obstruction of the biliary tract at cholangiography during operation Histological examination of a liver biopsy specimen removed at operation showed fatty degeneration and biliary stasis in one of the patients and hepatitis and biliary stasis in the other. The AST was elevated in both of them After precipitation of the β-lipoproteins the AST fell to 35 units, in the former case, and to 210 units in the latter One patient with severe acute cardiac failure—later post mortem showed widespread unicellular necrosis of the liver—had an AST of 425 units, which fell to 65 —A marked decrease of the titre was noted in the patient with biliary cirrhosis In the other patient with cirrhosis and yellow serum the AST was only moderately increased (425 units) and it fell to normal level In all 3 patients with cirrhosis and serum of normal gross appearance the AST fell distinctly after precipitation of the β lipoproteins, though in one not quite to the normal range (6000→300 units)

TABLE 3
AST before and after Precipitation of β-Lipoproteins in Dieases Secondary to Streptococcal Infection

Diagnosis	AST before	AST after
	precipitation of β lipoproteins	
Rheumatic fever	5000	3000
	2500	1000
”	1000	720
Acute tonsillitis	1250	850
	1000	600
	850	500
Acute nephritis	850	425

Table 3 gives the AST before and after precipitation of the β-lipoprotein in diseases secondary to streptococcal infection The titer decreased in all the fall was, however, only slight to moderate and never to normal

DISCUSSION

Judging from the findings the AST increase common in jaundice is invariable and marked in extrahepatic and intrahepatic cholestasis After precipitation of the β-lipoproteins, which occur in high concentration in cholestasis, the streptolysin inhibiting power of the serum markedly decreased and the AST fell to normal or almost normal level

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THE RELATIONSHIP OF PROPERDIN LEVELS TO THE BACTERICIDAL ACTIVITY OF HUMAN SERA

By

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Received 26 IX 62

Pillemer et al (5) described the properdin system consisting of the following components properdin, complement and magnesium ions, and presented investigations suggesting a close connection between this system and natural resistance towards bacterial infections Following the publication of *Wardlaw & Pillemer* (9) where they proposed that "The properdin system is the mechanism by which certain bacteria are destroyed by fresh normal human serum", an increased interest appeared concerning the normal bactericidal activity of serum These authors also stated that A quantitative relationship exists between the final properdin concentration and bactericidal activity against sensitive organisms An investigation of the kinetics of the bactericidal activity of the properdin system (10) showed however, that there was no simple correlation between properdin concentration and bactericidal activity

Wallers (11) found that elimination of properdin did not abolish the bactericidal properties of most human sera Bactericidal activity against Gram negative organisms was often observed in sera (reagents) or combinations of reagents not containing properdin These results of Wallers throw serious doubt on the proposed importance of properdin with regard to the bactericidal activity of fresh sera

The above mentioned disagreements need clarifying The question should also be reevaluated keeping in mind that there seems to be three different patterns of properdin levels one at which the persons show consistently high levels one with consistently low levels, and a third group in which the properdin titre changes during an infection (1)

For these reasons and also because it has been suggested that the factor of importance is not the properdin content as such, but the "available properdin or even unknown inhibitors or cofactors (3, 8 12) we engaged in the present study Accordingly, in addition to

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β-lipoproteins secondary to biliary stasis By means of a simple pre cipitation process—with dextran sulphate and calcium chloride—the β-lipoproteins can be separated off, and then this type of AST falls markedly while a specific titre is influenced but slightly

Addendum—After this article had gone to press I have noticed a report of non specific AST in diseases of the liver in Ann Biol clin *20* 525 1962 *Balin et al* show the importance of the β lipoproteins and adopt the dextran precipitation technic too

REFERENCES

Badin J & *Cabau N* Les fausses antistreptolysines Path Biol 7 1559 1959

Burstein M & *Samaille J* Sur une nouvelle méthode de dosage du cholestérol lie aux α et aux β lipoprotéines du serum Clin chim Acta *3* 320 1958

Hewitt L F & *Todd E W* The effect of cholesterol and of sera contaminated with bacteria on the haemolysins produced by haemolytic streptococci J Path Bact *49* 45 1939

Kalbak K The antistreptolysine reaction technic The State Serum Institute Denmark Copenhagen 1947

Oker Blom N *Nikkilä E* & *Kalaja T* Non specific antistreptolysin reactions in hepatitis sera Ann Med exp Fenn *28* 125 1950

Packalén T Nonspecific antistreptolysin reactions and serum cholesterol J Bact *56* 143 1948

[illegible] & *Petersmann G* Untersuchungen uber die Bedeu [illegible] Antistreptolysintiter Klin Wschr *35* 412 1957

[illegible]*oller F* & *Schon H* Untersuchungen uber die [illegible] und Proteinfraktionen des Serums als Strepto [illegible] *37* 1037 1959

Sievers O Antistreptolysintitreringar vid laboratoriet i Goteborg Svenska Lak Tidn *44* 1837 1947

Stollerman G The use of the hemolysin inhibition in the study of experimental and clinical hyperlipemia the nonspecific inhibition of streptolysin O by serum lipoproteins J clin Invest *33* 1233 1954

Waldenstrom J & *Winblad S* Some observations on the relationship of certain serological reactions in various diseases with hypergammaglobulinemia Acta rheum scandinav *4* 3 1958

Westergren A A clinical study of anti streptolysin titres in acute pleurisy Acta med scandinav Suppl *213* 323 1948

globulinaemia and extremely low resistance to infections Here, contrary to all expectations, a *high* properdin level in serum was found The titre was determined repeatedly and with all the three methods mentioned above We can offer no explanations of this unexpected finding

In some of the cases tested, the properdin titres rose from low values in the acute phase of an infectious disease, to normal values during convalescence Not even in these cases was it possible to demonstrate any changes in the bactericidal activity of serum

Figs 1 and 2, presenting two cases of pneumonia illustrate this very clearly

Table 2 shows a comparison of the bactericidal activity of serum and the properdin content when determined with the various methods

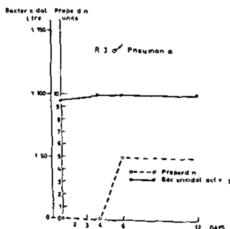


Fig 1

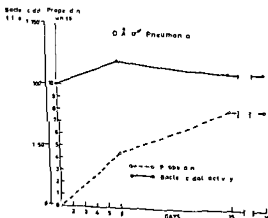


Fig 2

Pillemer's method for properdin titrations, we also used, in some experiments, the modifications by *Willers* (11), and by *Leon* (3), as these modifications presumably take the questions of inhibitors and/or cofactors into account

MATERIAL AND METHODS

Samples for parallel determinations of properdin and bactericidal activity were drawn from 52 patients with various complaints, and from 4 healthy persons

The blood samples were allowed to clot at room temperature rimmed and centrifuged The sera were kept at —20° C in small portions to cover one experiment each All samples were treated under sterile conditions

Pillemer's method for properdin titrations (5, 6) was applied to all of the samples and in addition to this some samples were also assayed according to *Willers* (12) and *Leon* (3) The detailed technique for the properdin titrations and a comparison of the three methods, have been described in an earlier publication (14)

The test for bactericidal activity used througout this study was as follows The test microbe employed was a serum susceptible strain of *Shigella dysenteriae* It was a laboratory strain (Sonne III), agglutinating specifically with antiserum to its species It was kept on Dorset's egg medium at —20° C for use as a stock culture An 18 20 hour subculture in brain heart infusion broth was used as inoculum in the actual test

The serum samples were diluted with physiological saline in twelve dilutions 1/10, 1/20–1/140 each tube containing 1 ml of the dilution To each tube was added 1 drop of undiluted inoculum and the rack was incubated at 37° C After 3 hours and again after 24 hours a loopful of each dilution was plated on nutrient agar plates The plates were read after 24 hours and the endpoint of the titration was taken as the last dilution giving no growth or just a few colonies The results given are those obtained after 3 hours inoculation before plating

The bactericidal activity of sera from normal, healthy persons determined by this method ranged from 1/70–1/120 the mean value (reciprocal value of the dilution) being 96

RESULTS

In Table 1 the sera are divided into three groups according to their properdin titres, and the mean values and range are given for the corresponding bactericidal titres

TABLE 1

Comparison of Properdin Levels and Bactericidal Activity of Serum

Group	Properdin units pr ml serum	Bactericidal titre	Lowest and highest bactericidal titre
Low properdin levels	< 1	91	50 140
Normal properdin levels	1 5 5	102	70 140
High properdin levels	> 6	94	20* 140

* A patient with hypogammaglobulinaemia

Whether the sera have no detectable properdin as determined by the Pillemer technique, or the properdin level is normal or even high, the bactericidal activity is similar and within normal range with the exception of one sample A noteworthy finding is that of the very low bactericidal activity of this serum from a patient with hypogamma-

The work of *Osawa & Muschel* (4) also clearly indicated that the properdin system itself is inadequate to explain the bactericidal activity of normal serum When human serum was absorbed with the test organism, it was inactive despite the presence of properdin and complement

It may be argued that *Wardlaw & Pillemer* (9) have shown that one unit of properdin pr ml of serum is sufficient for maximum bactericidal activity, and that sera containing less than one unit are difficult to titrate with accuracy Accordingly, they may contain just under one unit of properdin, and still have enough for normal bactericidal activity This is unlikely however, as some experience with properdin titrations quickly suggests that several normal sera contain no properdin at all or in any case considerably less than one unit pr ml

All evidence points to the fact that the properdin system is not a basic factor in the complex which controls man's resistance to microbial infections and more specifically, it is not as suggested by *Wardlaw & Pillemer* (9) the mechanism by which certain bacteria are destroyed by fresh sera

SUMMARY

The bactericidal action of human sera was compared to the properdin levels In three groups of persons, one showing consistently high properdin levels, one showing consistently low properdin levels, and one where the properdin titre rose from low values in the acute phase of an infection, to normal values during convalescence, no correlation could be found between these properdin titres and the bactericidal activity of the sera

REFERENCES

1 *Boe J & O* [illegible] The P [illegible]
tious [illegible]
2 *Hin C F* [illegible]
Acad
3 *Leon M A* Quantitative Studies on the Properdin Complement System J Exp Med *103* 285–293 1956
4 *Osawa E & Muschel L H* The Bactericidal Action of Normal Serum and the Properdin System J Immunol *84* 203 212 1960
5 *Pillemer L Blum L Lepow I H Ross O A Todd E W & Wardlaw A C* The Properdin System and Immunity I Demonstration and Isolation of a New Serum Protein Properdin and Its Role in Immune Phenomena Science *120* 279 285 1954
6 *Pillemer L Blum L Lepow I H Wurz L & Todd E W* The Properdin System and Immunity III The Zymosan Assay of Properdin J Exp Med *103* 1 13 1956
7 *Rowntree R J & Rantz L A* A Study of the Relationship of the Normal Bactericidal Activity of Human Serum to Bactericidal Infection J Clin Invest *39* 72-81 1960
8 *Soulier J P & Ménaché D* Variations pathologiques du complement et de la properdine chez l'homme Acta Haemat *20* 260 274 1958
9 *Wardlaw A C & Pillemer L* The Properdin System and Immunity, V The Bactericidal Activity of the Properdin System J Exp Med *103* 553 575, 1956

The sera have been arranged according to the increase in properdin titre assayed with Pillemer's method, and no correlation to the bactericidal activity is revealed when considering either of the three properdin titres for each serum.

TABLE 2
Properdin Levels Determined by three Different Methods as Compared to Bactericidal Activity

Serum number	Diagnosis	Bactericidal titre	Properdin units pr. ml serum		
			Pillemer's method	Willers method	Leon's method
1	Akromegalia	70	< 1	3	7
2	Vitium cordis arterioscleroticum	50	< 1	7	7
3	Colitis ulcerosa	80	< 1	12 – 14	> 8
4	Febris rheumatica	70	1.5	12	8
5	Healthy person	110	4	5	4
6	Leukemia acuta	90	4	8	8
7	Hypogammaglobulinaemia	20	8	8	8
8	Cirrhosis hepatis	70	8	12 – 16	> 8

DISCUSSION

It seems clear that there is no close connection between the bactericidal activity of serum and the properdin level whichever method is used for the properdin determination.

In agreement with *Roantree & Rantz* (7) we found very small variations in the bactericidal activity of human sera whether from normal or pathological subjects.

Hinz (2) found that in some patients with pneumococcal pneumonia, meningococcemia and pyelonephritis, the properdin titre would rise from low values in the acute stage, to normal values during convalescence. In the last group he also found increasing bactericidal activity with increasing properdin titre.

We were not able to demonstrate any such correlation when an increase in the properdin titre occurred during an infection, (Figs. 1 and 2), nor was it possible to discover any connection between bactericidal activity and properdin content where the latter showed consistently high or consistently low values during an infection.

Wood et al. (13) investigated serum antibacterial activities in patients showing hyper-, hypo-, and agammaglobulinaemia and found normal bactericidal activity with the exception of two patients with agammaglobulinaemia, in whom *increased* bactericidal levels and normal properdin levels were found. These patients, however, had recently recovered from upper respiratory infections.

In our present investigation, one patient with hypogammaglobulinaemia and low resistance syndrome showed *low* bactericidal activity and high (normal) properdin titres.

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HYALURONIC ACID PRODUCTION BY A STRAIN OF STREPTOCOCCUS FÆCALIS ISOLATED FROM URINE

By

ARVE LYSTAD and P. E. JONER

Received 9.x.62

Apart from a record by *Henriksen & Joner* (1962) we have been unable to find any record concerning production of hyaluronic acid by strains belonging to the Enterococci. Production of hyaluronic acid by streptococci belonging to Lancefield's group A and C is well known. Recently a hyaluronic acid producing strain belonging to Lancefield's group D was isolated from a patient's urine.

MATERIALS AND METHODS

The strain was isolated from the urine of a medical student by the method of [illegible]

[illegible] of urinary tract infection.

Serological identification of the strain was made according to *Mackie & McCartney* (1960) after acid extraction of group specific polysaccharide. The sera A, C, and D were used in a precipitation test. The same extract was tested against antisera of group A through O by a gel diffusion technique (*Omland*).

Isolation and analysis of capsul[illegible]
broth containing 0.5 per cent glu[illegible]
centrifuged in the cold. The supe[illegible]
concentrated to about 1/10 volum[illegible]
Heidelberger & Dawson (1937) [illegible]
[illegible]
inf[illegible]
[illegible]
me[illegible]
0.9 [illegible]
pre[illegible]
Me[illegible]

Chromatography. About 20 mg [illegible]
3 N H[illegible]
after [illegible]
Flow[illegible]
acetat[illegible] ethyl [illegible]) Staining was done with aniline oxalate and ninhydrin.

RESULTS

After 24 hours' growth on blood agar plates at 37° C in a humid athmosphere, the colonies were very small and dry. After 48 hours' growth, the colonies became strongly mucoid, confluent, and non-hae-

10 *Wedgwood R J & Pillemer L* The Kinetics of the Bactericidal Activity of the Properdin System Ann N Y Acad Sci *66* 247 250 1956
11 *Willers J M N* Investigations on the Significance of the Properdin System for the Natural Resistance to Infections Drukkerij en Uitgeverij Lab r Utrecht 1958 1 148
12 *Willers J M N Pondman K W & Winkler K C* An Improved Properdin Titration Based on the Elimination of an Inhibitor Vox Sang 4 21–32 1959
13 *Wood D C Burgess L & Morginson W J* Serum Antibacterial Activity in Patients with Acute and Chronic Bacterial Infections and with Hyper Hypo and Agammaglobulinemia Lab Investig *10* 381 387 1961
14 *Oesthus O & Boe J* The Assay of Properdin Scandinav J Clin & Lab Investig *13* 14 22 1961

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MATERIALS AND METHODS

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Serological identification of the strain was made according to *Mackie & McCartney* (1960) after acid extraction of group specific polysaccharide. The sera A, C and D [illegible]

[illegible] preparation [illegible]

Viscosity measurements. The time of [illegible] meters of a 0.1 per cent polysaccharide [illegible] 0.9 per cent NaCl was measured with a [illegible] preparation. The hyaluronidase was pr[illegible] *Meyer et al.* (1951

Chromatography. About 20 mg polysaccharide portions were hydrolysed with 3 N H_2SO_4 at 100° C for 6 hours. Chromatography was carried out on the supernate after neutralizing with $BaCO_3$. The chromatograms were run for 17 hours using the following solvent systems: pyridin-ethyl acetate water (2:5:5) and pyridin ethyl acetate acetic acid water (5:5:1:3). Staining was done with aniline oxalate and ninhydrin.

RESULTS

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12 Willers J M N Pondman K W & Winkler K C An Improved Properdin Titration Based on the Elimination of an Inhibitor Vox Sang 4 21-39 1959
13 Wood D C Burgess L & Morginson W J Serum Antibacterial Activity in Patients with Acute and Chronic Bacterial Infections and with Hyper Hypo and Agammaglobulinemia Lab Investig 10 381 387 1961
14 Oisthus O & Bøe J The Assay of Properdin Scandinav J Clin & Lab Investig 13 14 22 1961

TABLE 1

Analyses of Capsular Polysaccharide

Type of analysis	Own analyses	Figures from literature
$(\alpha)_{D}^{20}$	—65°	—47° —78°
N	3 4 %	3 – 3 8 %
P	0 08 %	0
Acetyl	8 4 %	9 5 11 %
Precipitation of horse serum	+	+
Content of ash	2 3 %	4 % (5)

The yield of polysaccharide from the mucoid strain was 0 434 g The product was a pure grey white substance which gave highly viscous solutions in water Table 1 shows the analytical results

A corresponding preparation from a strain of a non mucoid Streptococcus fæcalis yielded 0 246 g

Table 2 shows the results of the viscosity measurements of the polysaccharide from the mucoid strain with Ostwald s viscosimeter OF I and OF II

TABLE 2

Examination of Viscosity of the Polysaccharide

Apparatus	Time of outflow without hyaluron dase	Time of outflow with hyaluron dase
OF I	11 8 sek	11 3 sek
OF II	2 min 17 4 sek	2 min 6 3 sek

Chromatography of hydrolysed polysaccharide isolated from the mucoid streptococcus gave the following results 1 Spots corresponding to glucosamine and glucuronic acid were detected 2 Additional spots corresponding to galactose glucose fucose and galacturonic acid (uncertain) were also present in the chromatograms

Chromatographs of the hydrolysed polysaccharide from the non mucoid streptococcus showed the presence of spots corresponding to galactose glucose fucose and galacturonic acid (uncertain) only

DISCUSSION

The strain differed from the typical Streptococcus fæcalis in several respects (*Bergey* 1957) poor growth on some simple media low temperature resistance failure to grow at 10° C and 45° C and in 6 5 per cent NaCl and delayed lactose fermentation But the points of resemblance including fermentation of mannitol and sorbitol and growth on 40 per cent bile and on tellurite agar are sufficient to suggest relationship to

molytic No growth was observed on bromthymolblue lactose agar plates The size of the colonies on blood agar was very variable, and the colonies became flattened and dry within few hours in a dry athmosphere

By direct microscopy of a Gram-stained smear from the urine, we found round to ovoid Gram positive cocci which occurred in short chains and in pairs After examining at least 20 fields, bacteria could be found in any microscopic field Microscopy from a culture, gave the same result The strain was found to be non-motile

The microbes were surrounded by rather narrow, but distinct, capsular spaces of variable size in india ink preparations (*Bull et al*, 1936)

The growth in broth culture was very scanty after 24 hours, but turbid after 48 hours There was no growth in phosphate broth without glucose The best growth was observed in broth containing 0 5 per cent glucose

Biochemical properties Acid was produced from maltose and glucose within 24 hours, from mannitol, sorbitol, trehalose and salicin within 48 hours, and from lactose within 5 days Sucrose, inulin and raffinose were not fermented and gelatin was not liquefied within 5 days No reduction appeared in skim milk with 0 02 per cent methylene blue within 5 days

The strain was resistant (*Ericsson et al*, 1954) to sulphonamides, penicillin, streptomycin, and doktacillin (penbritin) It was sensitive to chloramphenicol, oxytetracyclin, erythromycin, oleandomycin, and nitrofurantoin

The strain did not grow in 6 5 per cent NaCl in broth, but grew well on 40 per cent bile blood agar and on Mc Leod's tellurite medium within 2 days It survived heating to 56° C for half an hour but did not survive heating to 60° C for half an hour or heating to 56° C for one hour There was no growth within 48 hours in 1 per cent glucose in broth or on blood agar plates at 45° C, nor in the same media at 10° C Observing no growth at these temperatures, we incubated the cultures at 37° C in humid athmosphere and observed the usual growth as described before

The growth was good on blood agar plates with pH 9 6 within 48 hours The terminal pH in glucose in broth was 4 3 after 3 days growth The strain was resistant to optochin

Detection of hyaluronic acid After cross-streaking a hyaluronidase producing staphylococcus and our strain on blood agar, the growth of the strain was non-mucoid for a distance of about 1 cm from the staphylococcus streak

A precipitation test with the acid-extracted antigen was positive with Lancefield's group D antiserum but negative with the group antisera A and C In the gel precipitation test the extract reacted with the group antiserum D, but failed to react with the oher sera from group A through O

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THE INFLUENZA VIRUS HAEMAGGLUTINATION INHIBITION BY ANTIBODY TO HOST MATERIAL

By

ARILD HARBOE

Received 6 xii 62

Knight showed that rabbits injected with normal chick allantoic material produced antibody which inhibited the haemagglutination by influenza virus grown in chick embryos (18) Haemagglutination inhibiting (HI) antibody reacting with the viral host material was also demonstrated in persons given an injection of ordinary influenza vaccine (10) and in ferrets which repeatedly had received intranasal instillations of chick allantoic fluid infected with influenza virus (8) The detection of antibody to host material was facilitated by the use of purified virus in the HI test, and it was shown that the antibody was neutralized when normal allantoic fluid was added (8, 10) This paper presents a more comprehensive study of the HI antibody to host material

MATERIALS AND METHODS

Viruses The ex er ment
them
differ

By allantoic virus is meant virus which has been obtained from the allantoic fluid of inoculated chick turkey or duck embryos By mouse lung virus is meant virus obtained from tracheo bronchial washings or lung suspensions from intra nasally inoculated white mice The chick embryos were 10 days the turkey and duck embryos 12 13 days old when they were inoculated this took place intra allantoically Influenza B strains were harvested after 3 days the other strains after 2 days of incubation usually at 35° C. The normal allantoic fluids were harvested from embryos of the same age as the infected ones

The virus eluates were prepared by adding 1 per cent packed red cells to the chilled virus preparations The suspension was left for ½ hour at 0° C, subsequently the cells were spun down and the virus eluted into normal saline at 37° C for 1 2 hours Except when otherwise stated fowl cells were used

The author is indebted to Mrs *G Boe* Mrs *B Egeland* *sdal* and Mr *A Bye Hansen* for skilled technical assistance

the fæcalis group, and in addition the strain belongs to Lancefield's group D

The strain seems to produce hyaluronic acid capsules The chromatographic results seemed to indicate that the polysaccharide was contaminated by another polysaccharide containing galactose, glucose, fucose and, possibly, galacturonic acid However, our analyses of the polysaccharide isolated from the non-mucoid streptococcus showed that this strain also produces a polysaccharide that contains galactose, glucose, and fucose while the mucoid strain produces hyaluronic acid in addition

The spot corresponding to galacturonic acid may be due to a disaccharide, as hyaluronic acid seems to be partly resistant to acid hydrolysis

Henriksen & Joner (1962) found the same sugars from the capsule extract of their Streptococcus zymogenes by chromatography They presumed that their streptococcus product was a mixture of hyaluronic acid and another polysaccharide Our investigations probably explain the occurence of these sugars

By way of conclusion it must be added that the litterature from recent years includes a description only by *Henriksen & Joner* (1962) of isolation of a hyaluronic acid producing enterococcus strain There can be no doubt that the strain here described also is a hyaluronic acid producing strain

SUMMARY

A strongly mucoid strain of atypical Streptococcus fæcalis is isolated from the urine of a patient with urinary tract infection The investigations show that the strain is producing hyaluronic acid

REFERENCES

1 *Bergey* Manual of Determinative Bacteriology 7th ed 1957
2 *Butt E M Bonynge C W & Joyce R L* The demonstration of capsules about hemolytic streptococci with india ink or azo blue J infect Dis 58 5 9 1936
3 [illegible] *& Olmstead M* Variation in the hemolytic strepto [illegible] 38
4 [illegible] A paper disc method for determination [illegible] ierapeutic and antibiotic agents Scand J Clin Lab Invest 6 suppl *11* 23 25 1954
5 *Henriksen S D & Joner P E* Production of hyaluronic acid by a strain of Streptococcus zymogenes Acta path et microbiol scandinav *56* 70–73 1962
6 [illegible] clin Med *56* 899 907 1960
7 [illegible] A serologically inactive poly [illegible] of group A hemolytic strepto [illegible]
8 [illegible] 10th ed 1960
9 [illegible] iction of monosaccharides from [illegible] Chem *192* 275 281 1951
10 *Omland T* Personal communication

antigen in the normal allantoic fluid, and it is seen that this antigen is active in rather high dilutions in the HI test. Also HI antibody found after immunization with normal chick chorioallantoic membrane was neutralized by the normal allantoic fluid antigen. In the following sections the author uses the terms "host specific" and "virus specific" antibodies. Host specific antibody, as will be shown below, is produced when the host material is administered, whether virus is present or not, and is characteristic only of this material. Virus specific antibody is produced only when virus material is administered, and is characteristic of the virus only.

TABLE 1

Haemagglutination Inhibition Test with a Red Cell Eluate of Chick Allantoic PR-8 and a Cholera Filtrate treated Serum from a Rabbit repeatedly Injected i.v. with a Normal Chick Allantoic Fluid. Serial Dilutions of an Untreated Normal Chick Allantoic Fluid Were Added to Serial Serum Dilutions in a Chessboard Titration

Dilution of the normal chick allantoic fluid	Number of the haemagglutinating virus doses in the presence of the diluted normal allantoic fluid	Serum titres adjusted to 4 haemagglutinating virus doses
5	3	<7.5
10	4	<10
20	7	20
40	12	45
80	12	60
160	12	90
320	12	120
640	12	180
2560	12	210
saline only	12	240

2. *Host Specific HI-Antibody after Injection of Virus Infected Material*

In rabbits which had received injections of virus infected, chick allantoic fluid or purified, chick allantoic virus host specific HI-antibody could be demonstrated by testing the sera with a chick allantoic v specific antibody to the following injections of c PR 8 could be demonstrated when the sera were tested with chick allantoic Lee, a B strain, provided the latter virus was free from allantoic fluid. That this inhibition was due to host specific antibody and not to minor crossings between virus specific antigens (9), or to any contamination of the injected material with a B-strain, was demonstrated by the inhibition neutralizing effect of the normal chick allantoic fluid, and by the absence of inhibition when the sera were tested with a virus grown in a different host, see Table 2 (see also Section 6). It was checked that the duck allantoic Lee was not less sensitive than the chick allantoic Lee to homologous, virus specific antibody. The last one of the sera in the table was in addition tested with mouse lung Lee, and gave the

Immune sera In a single experiment (Section 9b) convalescent ferret sera were examined The other experiments were performed with sera from rabbits which had received several intravenous injections of the antigen concerned Usually the rabbits received 6 injections within one month the immune serum was drawn about a week after the last injection Booster doses were sometimes given Except when otherwise stated the sera were treated with cholera filtrate (Philips Duphar) in order to destroy normal inhibitors (28), and absorbed with 10 per cent fowl red cells in order to remove serum haemagglutinins

HI-test This was performed in the World Influenza Centre 'Perspex plates as previously described (15) The antibody titres correspond to 4 haemagglutinating doses of the virus When HI antibody to host material was titrated a red cell eluate of the virus was always employed The diluted eluates used as antigens were found to be more stable when they had been made up in buffered saline instead of the normal one which otherwise was employed in these experiments One volume of 0 15 M potassium phosphate buffer pH 7 2 was added to 9 volumes of the 0 85 per cent saline

HI-test in the presence of normal allantoic fluid As a routine the fluid was first treated with an equal volume of cholera filtrate over night at 37° C followed by 1 hour at 56° C and absorption with 10 per cent packed fowl red cells for ½ hour at 0° C To each serum dilution in the HI test was added 0 25 ml of the pretreated allantoic fluid and the mixture left to stand ¼ hour on the bench at room temperature before the red cells and the virus were added (although experiments showed that 5 minutes would have been sufficient) In a parallel test normal saline only was added

RESULTS

1 *Evidence that the Antihaemagglutinin Resulting from Injections of Normal Host Material Is an Antibody*

First it was checked that this antihaemagglutinin was not a normal serum inhibitor The inhibitor reacting with PR-8 is the α (Francis)-inhibitor, which is heat resistant, but sensitive to the receptor destroying enzyme in cholera filtrate Normal rabbit serum was compared with serum drawn after injections of normal chick allantoic fluid In an HI-test against chick allantoic PR-8 the titre of the normal serum before treatment with the enzyme was 80, after treatment < 10 The titre of the immune serum, however, was unchanged 640 The increased content of antihaemagglutinin in the immune serum could therefore not be explained as due to an increase of normal serum inhibitor

Next it was examined whether the antihaemagglutinin resulting from injections of normal chick allantoic fluid would react *in vitro* with the fluid In a chessboard titration serial 2-fold dilutions of an untreated, normal chick allantoic fluid were added to serial 2-fold dilutions of an antiserum against the fluid, and left on the bench at room temperature for 2 hours Subsequently a 0 5 per cent fowl red cell suspension was added and immediately afterwards chick allantoic PR 8 All components were added in volumes of 0 25 ml The effect of the normal allantoic fluid inhibitor on the haemagglutinin was examined in a simultaneous titration The result is shown in Table 1 As usual the HI-titres presented have been adjusted so that they all correspond to 4 haemagglutinating doses The table shows that the normal allantoic fluid can neutralize the antihaemagglutinin which it had produced *in vivo* This antihaemagglutinin must therefore be an antibody to an

TABLE 3

Antibody Titres of Sera from a Rabbit Repeatedly Injected i v with one ml of a [illegible]

Day number	Titres or injection	Haemagglutination inhibiting antibody titres against chick allantoic PR-8	Haemagglutinating antibody titres against fowl red cells	Haemagglutinating antibody titres against guinea pig red cells
0	Titres	< 10	< 5	< 5
0 2, 4	Injected			
14	Titres	10	< 5	5
21, 23 25	Injected			
35	Titres	100	10	20
100	Titres	< 10	10	10
100	Injected			
107	Titres	480	40	20

This conclusion is in agreement with the result of a comparison between the HI-titre and the haemagglutinating serum titre at different stages during the immunization with *normal chick allantoic fluid* Table 3 shows that the former titre fluctuates more than the latter, indicating that HI antibody is different from the haemagglutinating As expected from these results an attempt to produce HI-antibody by injecting a rabbit *i v* with fowl red cells failed, although the haemagglutinating titre reached 5000

In another experiment fowl and human red cells were compared in titrations of host specific HI-antibody Anti chick allantoic Lee serum was tested with chick allantoic PR-8 Fowl and human red cells were used to prepare the eluates and to remove the serum haemagglutinins The results are shown in Table 4 It is seen that fowl and human cells recorded practically the same titre values Also fowl and guinea pig red cells were compared Second cycle eluates of chick allantoic PR-8 were prepared with either type of cells and tested with an antiserum against chick allantoic Lee When fowl cells were employed in the

TABLE 4

[illegible]

Eluate prepared with red cells from	Sera absorbed with red cells from	Haemagglutination inhibition test performed with red cells from	Normal serum titres	Immune serum titres
fowl	fowl	fowl	< 9	36
man	man	fowl	< 9	36
man	man	man	< 6	48

titre < 6 Table 2 shows that the addition of the normal allantoic fluid did not reduce the virus specific antibody titres, which were recorded with duck allantoic PR-8 In another test it was found that the allantoic fluid did not reduce the titres recorded with an eluate of chick allantoic PR-8 neither

It was found that rabbits which had received injections of virus infected, chick allantoic fluid or purified, chick allantoic virus, usually presented higher host specific HI-titres than the rabbits did, which had received normal chick allantoic fluid or chorioallantoic membrane suspension

TABLE 2

Haemagglutination Inhibition Test with Purified Viruses (Eluates) and Cholera Filtrate treated Sera from a Rabbit Injected Repeatedly i v with one ml of PR 8 Infected Chick Allantoic Fluids Also the Normal Allantoic Fluid Had Been Treated with Cholera Filtrate

Tested against	Chick allantoic Lee		Duck allantoic Lee plus saline only	Duck allantoic PR-8	
Date of titres and injection	Plus saline only	Plus normal chick allantoic fluid		Plus saline only	Plus normal chick allantoic fluid
Titres Nov 2 1959	< 9	< 9	< 6	< 9	< 9
Injected Nov 2 4 6 23 25 27 – 1959 Titres Dec 7 1959	576	9	< 6	4600	4600
Injected May 4 1960 and March 17 1961 Titres Dec 9 1961	72	< 9	< 6	1150	1150
Injected Dec 9 1961 Titres Dec 15 1961	2300	36	< 6	2300	2300

3 *Does Host Specific HI-Antibody React with the Virus or with the Red Cells?*

In order to answer this question the effect of treatment with fowl red cells on the HI-titre of an antiserum against normal chick allantoic fluid was first examined To the cholera treated serum was added an equal volume of a 15 per cent suspension of the red cells, and the mixture kept at 0° C for one hour Subsequently the cells were spun down in the cold, and the unabsorbed and absorbed sample tested with chick allantoic PR 8 It was found that they gave the same titre, 320 The titre of the corresponding normal serum was < 10 Also the sera in Table 2 were tested unabsorbed and absorbed These sera were treated twice with 10 per cent packed red cells for ½ hour at 0° C Also here the absorptions failed to reduce the HI-titres As regards the titres of the serum haemagglutinin they were quite low already in the unabsorbed sera (10 or less) The absorption(s) gave, however, distinct titre reductions It is concluded that the HI-activity of the host specific antibody can not be due to a reaction between serum and red cells, and the explanation must therefore be a reaction between serum and virus

the mouse line were tried Fowl and mouse red cells were used to prepare the eluates, to remove the haemagglutinins from the sera, and in the HI test The experiments did not reveal any haemagglutination inhibition of mouse lung virus by antibody to host material Neither was the ferret mouse strain Talmey, a human A-strain from 1937 which never had been passed in eggs, inhibited by an anti normal mouse lung serum

b *Virus grown in duck embryos* Immune sera were prepared against normal duck allantoic fluid, duck allantoic PR-8 and Lee The sera were HI tested with duck allantoic Lee and PR 8 Fowl and duck red cells were used to prepare the eluates, to remove the haemagglutinins from the sera, and in the HI-test The experiments did not show any haemagglutination inhibition of duck allantoic virus by antibody to host material

c *Virus grown in turkey embryos* Sera from one rabbit injected with normal turkey allantoic fluid and 5 rabbits with PR 8 infected turkey allantoic fluid or a turkey red cell eluate of turkey allantoic PR-8 were tested with a fowl cell eluate of turkey allantoic Lee All animals showed host specific HI antibody, although the amount was quite moderate in 3 of them (titre only 24), including the one which had received normal allantoic fluid Attempts to demonstrate host specific HI antibody in the latter animal by means of PR-8 failed The titres in one of the 2 rabbits with a good antibody development is shown in Table 5 The duck allantoic Lee was included as a control, see Sections 2 and 6

Strains which had been adapted to the chick embryo, grew to high titres in the duck and turkey embryos already in their first passage in these hosts, and this passage was employed in the experiments described

TABLE 5

Haemagglutination Inhibiting Antibody Titres of Sera from a Rabbit Injected with PR-8 Infected Turkey Allantoic Fluid Fowl Red Cells Were Employed also in this Experiment

Day number	Titres or injection	Tested with red cell eluates of				
		Turkey allantoic Lee		Duck allantoic Lee plus saline only	Turkey allantoic PR 8	
		Plus saline only	Plus normal turkey allantoic fluid		Plus saline only	Plus normal turkey allantoic fluid
0	Titres	< 6	< 6	< 9	< 9	< 6
0 2 6 21 23 25	Injected					
32	Titres	192	< 6	< 9	2300	2300

6 *Cross-Reactions of Host Specific HI-Antibodies*

Chick and turkey allantoic viruses were inhibited by antisera against chick, turkey and duck allantoic fluids infected with not-crossing strains The viruses were not inhibited by anti mouse lung sera As

HI-test, the two eluates recorded the same antibody titre, 400 When guinea pig cells were employed instead, both eluates recorded the titre 200 The results of these comparisons support the conclusion above, that it must be the virus which reacts with the serum

4 *Is the Host Antigen in the HI-Test an Accidental Contaminant or a Necessary Component of the Virus Particle?*

First it was examined whether additional cycles of adsorption and elution with fowl red cells might reduce the sensitivity of the virus to host specific HI-antibody It was then found that when 1st, 2nd and 3rd cycle eluates of chick allantoic PR-8 were tested against antiserum to normal chick allantoic fluid or chick allantoic Lee, there was practically no HI-titre difference between the eluates

In another experiment chick allantoic PR-8 purified by means of differential centrifugation (½ hour at 2 000 × G, 1 hour at 12 000 × G) was tested against antiserum to chick allantoic Lee and found to give nearly the same HI-titre as an eluate

Jensen pointed to the possibility that treatment of the virus suspensions with ether might release antigens or change antigenic structure in such a manner as to shed more light on the problem of the nature of the host-tissue components of influenza virus (16) In the following experiment the host specific HI antibody sensitivity of the haemagglutinin from ether disrupted virus particles was examined A second cycle eluate of chick allantoic PR-8 was treated with ether according to the method of *Hoyle et al* (14) by shaking the eluate with half its volume of ethyl ether for 5 seconds, then leaving the mixture for one hour in the w b at 37° C, shaking again for 5 seconds, leaving another hour in the w b and then spinning at 1 800 × G The aqueous layer, expected to contain released haemagglutinin as well as intact virus particles, was subsequently spun for 2 hours at 25 000 × G, a procedure which sediments the intact virus particles and leaves the free haemagglutinin in the supernatant In the latter no virus particles could be seen in the electron microscope The released haemagglutinin was subjected to an adsorption elution cycle When the untreated virus eluate and the free haemagglutinin were compared in HI-tests against anti normal chick allantoic fluid sera, the haemagglutinin gave even higher titres than the intact virus did, a finding which shows that the ether treatment had failed to remove the host antigen from the haemagglutinin A similar experiment with Lee virus particles was also negative

5 *Does Host Specific Antibody Inhibit the Haemagglutination of Virus Grown in other Hosts than the Chick Embryo?*

a *Virus grown in mouse lungs* Immune sera were prepared against normal mouse lung, mouse lung PR-8 and Lee The sera were HI-tested with mouse lung Lee and PR-8, of the latter strain both the egg line and

8. *Does the Sensitivity of the Virus to Host Specific HI Antibody Increase with the Adaptation of the Virus to the Host?*

The two PR 8 lines were compared as antigens in an HI test against sera drawn before and after injections of normal chick allantoic fluid. Chick allantoic viruses were used; of the mouse line the first passage in chicks. The egg line gave the HI titres < 15 and 60, the mouse line 30 and 180. A similar difference was observed when the viruses were tested with 7 anti chick allantoic Lee sera. Thus the egg line was not more sensitive than the mouse line to antibody against chick allantoic material.

In another experiment 17 successive chick allantoic passages of the strain A_2/Norway/1/58 were HI tested with rabbit antisera against chick allantoic PR 8 and Lee, and with convalescent ferret sera against A_2 Japan 305/57 FFME. The first passage tested was chick amniotic$_1$ allantoic$_3$; earlier passages were not available for this experiment. The rabbit sera had to be pretreated with periodate in order to destroy the normal inhibitor to these viruses (12). With the ferret sera the standard treatment with cholera filtrate (28) was sufficient. It was found that there was no substantial difference between the various passages as regards their sensitivity to host specific and virus specific HI antibodies. —A chessboard titration with serial 2 fold dilutions of the passages am$_1$al$_3$, am$_1$al$_4$ and am$_1$al$_{10}$ against serial 2 fold dilutions of anti chick allantoic Lee did not disclose any heterogeneity within the antigens as regards their sensitivity to host specific HI antibody.

9. *Host Specific HI Antibody and Antigenic Comparisons of Influenza Strains*

a. *Sera from immunized rabbits.* In a previous investigation it was found that some of the rabbits injected with chick allantoic A strains other than A_2 presented HI antibody to chick allantoic A_2-strains (12). It was suggested that this phenomenon most likely was due to host specific antibody which *had not* been completely neutralized by the periodate treated normal chick allantoic fluid added. In the present investigation 5 of the 8 sera concerned could be retested by means of duck allantoic A_2 Japan 305/57 FFME, namely anti swine influenza, Melbourne, Weiss, A_1 England 1/55 and A_1/Denver 1/57. As a duck allantoic virus the A_2-strain was insensitive to host specific HI antibody. To virus specific A_2-antibody however, it possessed a high sensitivity. It was found that the duck allantoic A_2-virus was not inhibited by the five sera.

b. *Sera from convalescent ferrets.* Four ferrets were given a single intranasal instillation of one ml of chick allantoic fluid infected with A_2 Japan 305/57 FFME. This resulted in detectable host specific HI antibody development in all four animals. The titres of the animal which presented the highest titres of host specific antibody are shown

shown in Table 6, turkey allantoic Lee failed to give a sharp endpoint when titrated with the antiserum against chick allantoic PR-8 In another test turkey allantoic PR-8 was not inhibited (titre < 5) by an antiserum against normal chick allantoic fluid, although the serum gave the titre 800 with chick allantoic PR-8 Mouse lung and duck allantoic viruses were not inhibited by antisera against chick, turkey and duck allantoic fluids or mouse lungs, whether normal or infected with not-crossing strains PR-8 and Lee were used in these experiments

TABLE 6
Haemagglutination Inhibition Test Showing Cross-Reactions between Host Specific Antigens and Antibodies

Rabbit antisera against PR-8 infected	Tested with fowl red cell eluates of				
	Chick allantoic Lee	Turkey allantoic Lee	Duck allantoic Lee	Mouse lung Lee	Duck allantoic PR-8
Chick allantoic fluid	4600	18 – 144	< 9	< 9	960
Turkey allantoic fluid	144	288	< 9	< 9	1440
Duck allantoic fluid	108	288	< 9	< 9	960
Mouse lung eluate	< 9	< 9	< 9	< 9	360

In a preliminary experiment fowl plague virus grown in chick embryonic skin tissue culture failed to be inhibited in an HI-test against an anti chick allantoic PR-8 serum, although the serum inhibited the chick allantoic fowl plague virus (see the following section)

Fowl, turkey and duck red cells compared in an HI-test performed with chick allantoic virus *versus* anti chick allantoic serum gave only minor titre differences

7 *Various other Strains Examined on their Sensitivity to Host Specific HI-Antibody*

The sensitivity of PR-8 and Lee was dealt with in the preceding sections In this section experiments with various other strains are described All of them had been adapted to the chick embryo and were tested as chick allantoic viruses The following strains were tested with an antiserum against normal chick allantoic fluid, and all gave a distinct inhibition Swine influenza virus and the human A-strains WS (1933), Melbourne (1935), Weiss (1943), FM-1 (1947), A1 England 1/51, A1/Persia/2/52, A1/India/31/55 A1/Italy/32/56, further the B-strains BON (1943), Crawley (1946), B/England/9 54, B/Netherlands/28/55 An antiserum against chick allantoic PR-8 gave a distinct inhibition of A2/Japan/305/57 EFME, A/Fowl plague (Rostock strain), A/Horse influenza (Prague), B Norway/1/61 and mumps virus (Enders strain), while A/Duck influenza (Czech strain), influenza C (Taylor's strain) and Sendai virus (HVJ) failed to show any inhibition

still unknown and in his HI tests the inhibitor therefore might have masked a moderate amount of host specific antibody

The author also demonstrated that the host specific HI antibody reacted with the virus and not with the red cells On the other hand various workers have shown that antisera against hostcells interfer with *infection* by means of cell antibody rather than virus antibody reactions (3 6 24 25)

A crucial problem is whether the host material is an accidental contaminant or a necessary building stone of the virus particle (16) Substantial amounts of host material are found also with highly purified myxoviruses Thus *Knight* found 20–30 per cent (19) *Cohen* 25 57 per cent (4) *Munk & Schafer* 42 per cent (23) There was no report on the content of host material in isolated haemagglutinin —Various authors have demonstrated a great similarity between the chemical composition of the virus and the corresponding normal host material certain differences have also been observed (1 5 13 17 18 20 21) *Frommhagen et al* wrote that it is difficult to escape the conclusion that host components are incorporated into influenza virus particles some time during their fabrication As regards the antigenic relationship *Knight* found that highly purified virus gave distinct cross reactions with normal host material in the precipitation test (19) and *Smith et al* did the same in the complement fixation test (26) These authors concluded that the host material or some closely related derivatives constituted an integral part of the virus particle *Ananthanarayan* arrived at the opposite conclusion in his complement fixation experiments the antigenically related host material found with the virus particle was probably a contaminant (2) He too however failed to prepare virus particles free from host material *Kroeger* showed that chick allantoic influenza virus could be purified so that it no longer contained anaphylactogenic material (22) *Springer & Tritel* found that ordinary influenza vaccine and chorioallantoic material from normal eggs contained bloodgroup A active substances (27)

The present author failed to reduce the host specific HI antibody sensitivity of the chick allantoic virus eluate by subjecting it to additional cycles of adsorption and elution with red cells Neither did the isolated haemagglutinin show a reduced sensitivity These negative results fail to disclose whether the host antigen is an extraneous contaminant or a functional part of the haemagglutinin They only show that the host antigen was not removed from the rest of the haemagglutinin by the methods employed However it appeared as if the host component was not incorporated into the haemagglutinating antigen of the *duck* allantoic viruses These viruses were not inhibited by antiserum against duck allantoic material although such serum inhibited the chick and turkey allantoic viruses

When sera from animals immunized with chick allantoic viruses were employed in strain studies the duck allantoic viruses appeared to

TABLE 7

Titrations Showing the Rapid Appearance and Decline of Haemagglutination Inhibiting Antibody against Chick Allantoic Material in a Ferret Infected by an Intranasal Instillation of one ml of A2/Japan/305/57 EFME Bearing Chick Allantoic Fluid

Serum drawn	Tested against chick allantoic			
	A Japan 305 57 egg line	A Japan 305 57 egg ferret mouse egg line	Lee eluate plus saline only	Lee eluate plus normal chick allantoic fluid
Before inoculation	< 6	< 6	< 9	< 6
7 days after inoculation	< 12	750	72	< 6
11 days after inoculation	12	3000	18	< 6
16 days after inoculation	12	1500	9	< 6
26 days after inoculation	18	500	< 9	< 6

in Table 7 It is seen that the host specific antibody reaches its maximum titre earlier than the virus specific antibody does, even when the latter is titrated with the antibody sensitive line of A_2Japan/305 57 (10, 11)

In a previous investigation the antigenic relationship between influenza strains was studied by means of cross-infected ferrets, which developed a more or less pronounced, occasionally no, increase of the HI-titre to the first strain in response to cross-infection with a second one (7) The antigens in the HI tests consisted of diluted, virus bearing chick allantoic fluids In the present investigation these sera were retested in order to see whether host specific instead of virus specific antibodies might have been responsible for some of the increases observed previously The new titrations of the A_2 antibodies were performed with duck allantoic A_2Japan/305 57 EFME, while the antibody titres to the other strains were determined with chick allantoic viruses in the presence of normal chick allantoic fluid In the new tests, which excluded host specific inhibition titre increases were found which were practically identical to those recorded in the previous investigation

DISCUSSION

Apart from a brief description in the paper by *Knight* (18) the author found no reports on the haemagglutination inhibition by antibody to normal host material In a subsequent paper *Knight* mentioned that when he tested highly purified preparations of chick allantoic Lee with antiserum to highly purified, chick allantoic PR-8, he found the serum to "lack ability to inhibit the agglutination of red cells to a significant extent compared with the homologous system (19) In the present investigation, however, distinct amounts of host specific HI-antibody was found also when virus eluates had been injected At the time of *Knight's* experiments specific methods to destroy normal serum inhibitor were

7 In addition to the human influenza strains, swine and horse influenza classical fowl plague and mumps virus were found to be sensitive to host specific HI antibody, while influenza C, duck influenza and Sendai virus were not

8 Attempts to increase the sensitivity to host specific HI-antibody by increasing the number of passages in the host concerned were negative

9 In ferrets even a single, intranasal instillation of influenza virus bearing chick allantoic fluid produced detectable amounts of host specific HI antibody, the titre peak came already within a week after the inoculation

REFERENCES

1 *Ada G L & Gottschalk A* The component sugars of the influenza virus particle Biochem J 62 686–689 1956

2 *Ananthanarayan R* The fabric of virus elementary bodies Brit J Exper Path 35 381 388 1954

3 *Borecky L & Zavada J* Effect of anticellular sera on infection with myxoviruses I The effect of antichorioallantoic serum Acta virol 4 110–123 1960

4 *Cohen S S* Cytoplasmic particles of chorio allantoic membrane and their relations to purified preparations of influenza virus Proc Soc Exper Biol & Med 57 358 360 1944

5 *Frommhagen L H Knight C A & Freeman N K* The ribonucleic acid lipid and polysaccharide constituents of influenza virus preparations Virology 8 176–197 1959

6 *Habel K Hornibrook J W Gregg N C Silverberg R J & Takemoto K K* The effect of anticellular sera on virus multiplication in tissue culture Virology 5 7 29 1958

7 *Harboe A* The haemaggl [illegible] with [illegible] microb [illegible]

8 *Harboe A* [illegible] fluenza [illegible] path et micr biol scandinav 55 425–436 1962

9 *Harboe A* HI antibodies to [illegible]

10 *Harb* [illegible]

11 *Harboe A & Reenaas R* The course of antibody development to influenza A- strains Examined in the haemagglutination inhibition test with viruses of different sensitivity to homologous antibody Acta path et microbiol scandinav 46 266–272 1959

12 *Harboe A Reenaas R & Juul K* Influenza A₀ strains against A and A₁ strains in cross haemagglutination inhibition tests Acta path et microbiol scandinav 48 231 242 1960

13 *Hoyle L & Davies S P* Amino acid composition of the protein components of influenza virus A Virology 13 53 57 1961

14 *Hoyle L Jolles B & Mitchell R G* The incorporation of radioactive phosphorus in the influenza virus and its distribution in serologically active virus fractions J Hyg 52 119 127 1954

15 *Isaacs A Gledhill A W & Andrewes C H* Influenza A viruses Laboratory studies with special reference to European outbreak of 1950–51 Bull World Health Organ 6 287 315 1952

16 *Jensen K E* Serological relationships among influenza viruses D Host tissue components of virus In Advances in virus research IV Academic Press Inc., New York 1957

be useful as antigens in the HI-tests because they were resistant to host specific antibody and at the same time as sensitive to virus specific antibody as the parent chick allantoic viruses were (8, 9)

The experiments failed to reveal any influence of adaptation on the sensitivity to host specific HI-antibody However, it remains to examine the sensitivity of the very first chick embryo passage of strains isolated in this host The question to arise is whether repeated passages in duck embryos might result in duck allantoic virus which is sensitive to its host specific HI-antibody

SUMMARY

1 Rabbits were injected i v with normal chick allantoic fluid and the sera examined in the haemagglutination inhibition (HI) test against an influenza virus grown in the chick embryo (chick allantoic virus) It was shown that the resulting antihaemagglutinin was an antibody, and this was called "host specific" in order to distinguish it from the antibody produced in response to a viral antigen, and which was called "virus specific" The often lowtitred, host specific HI-antibody was detected more easily when the virus antigen in the HI-test had been purified so that there was no allantoic fluid present to compete with the virus for this antibody The viruses were purified by means of absorption to and elution from red cells

2 Host specific HI-antibody resulting from injection of a chick allantoic virus could be demonstrated when in the titrations another chick allantoic virus was used, which was not inhibited by the virus specific HI-antibody produced in response to the injected one A suitable combination was a strain of the A type (PR-8) and the B-type (Lee)

3 The host specific HI-antibody could not be removed by absorption with red cells, neither was the titre correlated with the titre of the haemagglutinating antibody When fowl, human and guinea pig red cells were compared in the HI-test, nearly the same host specific antibody titre was recorded It was concluded that this antibody did not react with the red cells, and that it therefore must react with the virus in the HI test

4 Repeated cycles of absorption to and elution from fowl or guinea pig red cells, or disintegration with ether failed to reduce the sensitivity of the virus to host specific HI-antibody

5 Antisera against mouse lungs failed to inhibit the haemagglutination of viruses cultured in mouse lungs Neither were duck allantoic viruses inhibited by antisera against duck allantoic material Turkey allantoic viruses, however, were inhibited by antisera against turkey allantoic material

6 Both chick and turkey allantoic viruses were inhibited by antisera against chick, turkey and duck allantoic material Mouse lung and duck allantoic viruses were not inhibited by any type of host specific antibody

7 In addition to the human influenza strains, swine and horse influenza, classical fowl plague and mumps virus were found to be sensitive to host specific HI antibody, while influenza C, duck influenza and Sendai virus were not

8 Attempts to increase the sensitivity to host specific HI-antibody by increasing the number of passages in the host concerned were negative

9 In ferrets even a single, intranasal instillation of influenza virus bearing chick allantoic fluid produced detectable amounts of host specific HI antibody, the titre peak came already within a week after the inoculation

REFERENCES

1 *Ada G L & Gottschalk A* The component sugars of the influenza virus particle Biochem J *62* 686–689 1956

2 *Ananthanarayan R* The fabric of virus elementary bodies Brit J Exper Path 35 381 388 1954

3 *Borecký L & Zavada J* Effect of anticellular sera on infection with myxoviruses I The effect of antichorioallantoic serum Acta virol 4 110–123 1960

4 *Cohen S S* Cytoplasmic particles of chorio allantoic membrane and their relations to purified preparations of influenza virus Proc Soc Exper Biol & Med 57 358–360 1944

5 *Frommhagen L H Knight C A & Freeman N K* The ribonucleic acid lipid and polysaccharide constituents of influenza virus preparations Virology 8 176–197 1959

6 *Habel K Hornibrook J W Gregg N C Silverberg R J & Takemoto K K* The effect of anticellular sera on virus multiplication in tissue culture Virology 5 7 29 1958

7 *Harboe A* The haemagglutination inhibiting antibodies in ferrets crossinfected with influenza A_2 virus and strains of the other A types Acta path et microbiol scandinav *49* 224–236 1960

8 *Harboe A* An anamnestic antibody increase in ferrets cross infected with influenza A_2 virus after myxoviruses not belonging to the A group Acta path et microbiol scandinav 55 425–436 1962

9 *Harboe A* HI antib d es in fe [illegible]
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11 *Harbo*[illegible]

12 *Harb* [illegible]

13 *Hoyle L & Davies S P* Amino acid composition of the protein components of influenza virus A Virology *13* 53 57 1961

14 *Hoyle L Jolles B & Mitchell R G* The incorporation of radioactive phosphorus in the influenza virus and its distribution in serologically active virus fractions J Hyg 52 119 127 1954

15 *Isaacs A Gledhill A W & Andrewes C H* Influenza A viruses Laboratory studies with special reference to European outbreak of 1950–51 Bull World Health Organ 6 287–315 1952

16 *Jensen K F* Serological relationships among influenza viruses D Host tissue components of virus In Advances in virus research IV Academic Press Inc New York 1957

17 *Kates M Allison A C Tyrrell D A J & James A T* Lipids of influenza virus and their relation to those of the host cell Biochim et biophys acta *52* 455 466 1961
18 *Knight C A* A sedimentable component of allantoic fluid and its relationship to influenza viruses J Exper Med *80* 83 99 1944
19 *Knight C A* Precipitin reactions of highly purified influenza viruses and related materials J Exper Med *82* 281 294 1946
20 *Knight C A* The nucleic acid and carbohydrates of influenza virus J Exper Med *85* 99 116 1947
21 *Knight C A* Amino acid composition of highly purified viral particles of influenza A and B J Exper Med *86* 125 129 1947
22 *Kroeger A V* Evidence for the absence of anaphylactogenic host protein in highly purified PR 8 influenza virus J Immunol *89* 136 144 1962
23 *Munk K & Schafer W* Eigenschaften tierischer Virusarten untersucht an den Geflugelpestviren als Modell II Mitteilung Serologische Untersuchungen uber die Geflugelpestviren und uber ihre Beziehungen zu einem normalen Wirtsprotein Ztschr Naturforsch *6b* 372 379 1951
24 *Quersin Thiry L* Action de serums antitissulaires sur certaines infections a virus Thesis Brussels 1955
25 *Quersin Thiry L* Action of anticellular sera on virus infections I Influence on homologous tissue cultures infected with various viruses J Immunol *81* 253 260 1958
26 *Smith W Belyavin G & Sheffield F W* The host tissue component of influenza viruses Proc Roy Soc London ser B *143* 504–522 1955
27 *Springer G F & Tritel H* Blood group A active substances in embryonated chicken eggs and their relation to egg grown virus Science *138* 687 688 1962
28 *Veen J v d & Mulder J* Studies on the antigenic composition of human influenza virus A strains with the aid of the haemagglutination inhibition technique Onderz Meded Inst Praevent Gen No 6 Leiden 1950

FROM STATENS SERUMINSTITUT TREPONEMATOSES DEPARTMENT COPENHAGEN DENMARK AND THE VDT SECTION WORLD HEALTH ORGANIZATION GENEVE SWITZERLAND

EVALUATION OF THE FLUORESCENT TREPONEMAL ANTIBODY TEST (FTA)

By

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Received 6 ix 62

In January 1957 *Ad Harris W F Deacon & C A Smith* (10) reported in a brief communication that studies were planned to decide whether the fluorescent antibody technique could be applied to the sero diagnosis of syphilis

A publication followed shortly after this describing the technique and giving tables of the preliminary results (4)

During the next few years several publications appeared on the "fluorescent treponemal antibody test (FTA) as the new test was called

The FTA test was examined in the Treponematoses Department of Statens Seruminstitut Copenhagen as closer familiarity with this method was felt desirable considering its possible use in the program of research and though to a lesser degree—its possible routine use The department participated in two international collaborative studies on the specificity sensitivity and reproducibility of the FTA test These studies were under the leadership of *Ad Harris Chamblee* USA A report of the first study has appeared (25) while the second study has been concluded only recently and the report is in course of preparation

Previous Publications

The literature to date has been studied and a comparative table has been prepared of the various results obtained so as to elucidate the specificity and sensitivity of the FTA test A number of quantitative results are also recorded (Table 1) Table 3 is a brief schematic but practical review of the technique used in obtaining the results shown in Table 1 and Table 2

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TABLE 1

Comparison of Serological Results with FTA, TPI and STS from Previous Publications

Methods	FTA		Titer max	TPI		Titer max	STS		Titer max	Publication no	FTA		Titer max	TPI		Titer max	STS		Titer max
Reactivity R or non reactivity NR	R	NR		R	NR		R	NR		(see references)	R	NR		R	NR		R	NR	
	Primary syphilis untreated										*Primary syphilis treated*								
	12	2	2430	9	5	800	11	3	Kline 16 units	1	8	0		4	4		2	6	
Number of sera	8	2	3200	7	3		9	1		5	4	0	1600	4	0		0	4	
	80	49		69	61		88	42		19	19	32		28	23		7	44	§
	8	0		3	5				*	7	24	2		8	18				†
	22	0	2000	18	4		22	0		24	12	0	400	0	12		12		
										9	6	5		0	11				
	130 71%	53		106 59%	78		130 74%	46			73 65%	39		44 39%	68		21 28%	54	
	Secondary syphilis untreated										*Secondary syphilis treated*								
	16	0	65610	16	0	1600	16	0		1	3	0		2	1		0	3	
Number of sera	10	0	6400	9	1		10	0		5	5	0	3200	5	0		3	2	
	88	2		90	1		91	0		19									
	5	0	1600	5	0		5	0		24									
										9	4	1		0	5				
	119	2		120	2		122	0			12	1		7	6		3	5	
	Latent syphilis untreated										*Latent syphilis treated*								
Number of sera	11	0		11	0		11	0		1	5	1		5	1		3	3	
										7	35	2		30	7				
										24	9	0		9	0		3	6	
										9	3	0		0	3				
	11	0		11	0		11	0			[illegible]2 94%	3		44 80	11		6	9	

* Pr[illegible]ly few cases [illegible] † [illegible] cases [illegible] syphilis § [illegible] syphilis

TABLE 2

Comparison of Serological Results with FTA, TPI and STS from Previous Publications

Methods	FTA		Titer max	TPI		STS		Publication no	
Reactivity R / non reactivity NR	R	NR		R	NR	R	NR	(see references)	
				SIII (Dement paral Tabes CNS asympt S vascularis)					
	15	0	196830	15	0	15	0	1	(treated + untreated)
	28	0	25600	28	0	21	7	5	
	213	19		226	6	199	33	19	
umbers of sera	16	0		14	2	6	0	7	(treated + untreated) (10 sera not tested (STS))
	30	0		30	0	22	8	24	
	302	19		313	8	263	48		
	94%			98%		84%			
				Other diseases					
	0	4		0	4	4	0	1	
Numbers of sera	4	70		8	66	4	70	19	
	0	40		0	40	3	35	23	
	0	100		0	100	4	42	9	
	4	214		8	210	15	147		
	1 8%			3 7%		9.3%			
				Presumed normals					
	0	41		0	41			1	
	0	25		1	24	0	25	5	
Numbers of sera	4	340		19	327	1	345	19	
	1	56		0	57	0	57	7	
	0	465		0	465	0	465	24	
	5	927		20	914	1	892		
	0 5%			2 1%		0 1%			

Table 1 shows that in primary syphilis, the FTA test is more sensitive than the Treponema pallidum immobilization Test (TPI), while there is no significant difference in sensitivity between the two serological methods as far as the other stages of syphilis are concerned In the section of Table 2 showing the results with sera from cases regarded as diseases other than syphilis, the FTA test is found to have a lower percentage of reactivity than the TPI test, i e a greater specificity It might be mentioned in this connection however, that all the reactive sera in this portion of the table originate from a single publication among those mentioned The same is largely true of the results for presumed normal persons

MATERIAL AND METHODS

Antigen Treponema pallidum Nichols' strain extracted from rabbit testicular tissue in 0.85 per cent saline. The suspension was centrifuged for about 5 minutes at 2000 G to remove blood corpuscles, fragments of tissue and spermatozoa. The concentration aimed at was about 35×10^6 treponemes per ml. A suspension such as this could be used for several weeks if stored at 4° C. No preservative was added. The suspension could be lyophilized and used with satisfactory results.

Conjugate. Rabbit antihuman gamma globulin conjugated with fluorescein isothiocyanate. The dye stuff was supplied by Sylvana, New Jersey, USA and the rabbit antihuman gamma globulin was prepared in the physico-chemical department, Statens Seruminstitut. The conjugation was done according to the procedure described by *Borel & Durel* (1). In later preparations non-bound dye was removed after conjugation by means of a sephadex column (14), saving much time.

Phosphate buffered saline was used, pH 7.2 (as Difco Haemagglutination buffer).

Mounting medium: 1 part buffered saline plus 9 parts chemically pure glycerine.

The technique used was by and large that described in Serological Tests for Syphilis Manual 1959 (22).

a) a circle of about 1 cm diameter is drawn on an acetone cleaned slide by means of a diamond stylus. One drop of antigen is placed within the circle and air dried, preferably by warm air.

b) The slide with the dried antigen is immersed in acetone for 10 minutes and again air dried.

c) The antigen spot is covered with one drop of the serum to be examined. The serum was primarily diluted in buffered saline, most often in a dilution of 1:200, but in special experiments an undiluted serum was used as well as dilutions 1:10 and 1:200, no actual quantitative test was made. The serum was heated to 56° C for 30 minutes before use. On re-examination the serum was again heated to 56° C for 10 minutes. The slide with the antigen spot covered with serum was incubated for 30 minutes at 20-22° C in a closed humidified container to hinder drying.

d) Rinse with buffer saline.

e) Blot gently with filter paper.

f) One drop of conjugate diluted in accordance with the titre, most often 1:40, is placed on the serum treated antigen spot. Incubate for 30 minutes at 20-22° C, protected against drying up (as in step c).

g) Rinse with buffer saline.

h) Place one drop of mounting medium on the spot and cover with a cover slip.

i) Read within 1-2 hours, as the fluorescence is quickly weakened on storage even in a refrigerator.

It will be observed that the slide is not rotated during phases c) and f), that the test is carried out at room temperature (20-22° C) and that Tween 80 is not used.

The microscope used was a binocular Zeiss Standard microscope GFL 658632. Illumination was with an Osram bulb type HBO 200. Exciter filter BG 12. Barrier filter above no filter below. UV barrier filter. Magnification 500.

The results of the readings were recorded as follows:

No fluorescence	—	designated as non reactive
Very weak fluorescence	+	designated as non reactive
Clear fluorescence	++	designated as reactive
Strong fluorescence	+++	designated as reactive
Very strong fluorescence	++++	designated as reactive

All sera examined in the FTA test were also examined in 1) the TPI test using the technique described in (16) but with 40 per cent complement by volume, 2) the Wassermann complement fixation reaction using Mørch's modification with cardiolipin antigen CWRM, 3) Kahn's standard test KR and 4) Meinicke's clarification test MR (20).

The results of the TPI test are given as non reactive — or reactive +, while the results of CWRM and KR are given in degrees of strength, for example CWRM 11, KR 9, and the results of MR are given semi-quantitatively by —, ±, + or ++ (20).

TABLE 3

Information on techniques used

	Antigen						Sera inactivation		Sensitization procedure				Conjugate			Conditions during treatment by conjugate		Buffer pH	Mounting medium
	Condition				Fixation														
	Fresh	Stored at 4° C	Lyophilized	Air-dried	Heat	Acetone	Temp (° C)	Time (min)	Dilution	Time (min)	Rotation r p m	Temp (° C)	Isocyanate IC	Isothiocyanate (ITC)	Tween (T) 2%	Rotation r p m	Temp (° C)		
1	I	S		D*	H		56	30	1 10	30		20 25		ITC			20 25	7 2	+
5	I	S	L	D		A	56	30	1 5	30		20 25		ITC			20 25	7 2	+
									1 200	30	160	37		ITC	T	160	37	7 2	+
19	I	S		D	H		56	30	1 5	30	160	20 25	IC			160	20 25	7 2	+
23	I	S		D	H	A	64	30	undil	30		20 25		ITC			20 25	7 2	+
							or 56	30	1 30	30		20 25		ITC			20 25	7 2	+
7	F	S		D	H ?		56	30	1 10	30		20 25		ITC			20 25	7 2	+
24	F		L	D		A	56	30	1 100 1 200	30	100	37		ITC	T	100	37	7 2	+
9	I	S		D		A	62	30	1 30	30		37		ITC			37	7 0	+

* 37 40° C

MATERIAL AND METHODS

Antigen Treponema pallidum Nichols strain extracted from rabbit testicular tissue in 0.85 per cent saline The suspension was centrifuged for about 5 minutes at 2000 G to remove blood corpuscles fragments of tissue and spermatozoa. The concentration aimed at was about 35 × 10^6 treponemes per ml A suspension such as this could be used for several weeks if stored at 4° C No preservative was added The suspension could be lyophilized and used with satisfactory results

Conjugate Rabbit antihuman gamma globulin conjugated with fluorescein isothiocyanate The dye stuff was supplied by Sylvana New Jersey USA and the rabbit antihuman gamma globulin was prepared in the physico chemical department Statens Seruminstitut The conjugation was done according to the procedure described by *Borel & Durel* (1) In later preparations non bound dye was removed after conjugation by means of a sephadex column (14) saving much time

Phosphate buffered saline was used pH 7 2 (as Difco Haemagglutination buffer)

Mounting medium 1 part buffered saline plus 9 parts chemically pure glycerine

The technique used was by and large that described in Serological Tests for Syphilis Manual 1959 (22)

a) a circle of about 1 cm diameter is drawn on an acetone cleaned slide by means of a diamond stylus One drop of antigen is placed within the circle and air dried preferably by warm air

b) The slide with the dried antigen is immersed in acetone for 10 minutes and again air dried

c) The antigen spot is covered with one drop of the serum to be examined The serum was primarily diluted in buffered saline most often in a dilution of 1 200 but in special experiments an undiluted serum was used as well as dilutions 1 10 and 1 200, no actual quantitative test was made The serum was heated to 56° C for 30 minutes before use On re examination the serum was again heated to 56° C, for 10 minutes The slide with the antigen spot covered with serum was incubated for 30 minutes at 20 22° C in a closed humidified container to hinder drying

d) Rinse with buffer saline

e) Blot gently with filter paper

f) One drop of conjugate diluted in accordance with the titre most often 1 40 is placed on the serum treated antigen spot Incubate for 30 minutes at 20-22° C, protected against drying up (as in step c)

g) Rinse with buffer saline

h) Place one drop of mounting medium on the spot and cover with a cover slip

i) Read within 1 2 hours as the fluorescence is quickly weakened on storage even in a refrigerator

It will be observed that the slide is not rotated during phases c) and f) that the test is carried out at room temperature (20 22° C) and that Tween 80 is not used

The microscope used was a binocular Zeiss Standard microscope GFI 658632 Illumination was with an Osram bulb type HBO 200 Exciter filter BG 12 Barrier filter above no filter below UV barrier filter Magnification 500

The results of the readings were recorded as follows

No fluorescence	—	designated as non reactive
Very weak fluorescence	+	
Clear fluorescence	++	designated as reactive
Strong fluorescence	+++	
Very strong fluorescence	++++	

All sera examined in the FTA test were also examined in 1) the TPI test using the technique described in (16) but with 40 per cent complement by volume 2) the Wassermann complement fixation reaction using Mørch s modification with cardiolipin antigen CWRM 3) Kahn s standard test KR and 4) Meinicke s clarification test MR (20)

The results of the TPI test are given as non reactive — or reactive + while the results of CWRM and KR are given in degrees of strength for example CWRM 11 KR 9 and the results of MR are given semi quantitatively by — ± + or ++ (20)

in the treated cases there is agreement between FTA-200 and TPI and disagreement between these and STS the reagin reactions giving fewer reactive cases

The 35 cases of Table 9 may be compared to the last group in Table 8 but here there is disagreement between FTA and TPI 200

TABLE 6

Secondary Syphilis 41 Untreated Cases

Disagreement between TPI and FTA_{200}						Agreement between TPI and FTA_{200}					
STS		TPI		FTA_{200}		STS		TPI		FTA_{200}	
	-		+	−	+		+		+	−	+
	1	1			1		40		40		40

TABLE 7

Secondary Syphilis 21 Treated Cases

Disagreement between TPI and FTA_{200}						Agreement between TPI and FTA_{200}					
STS		TPI		FTA_{200}		STS		TPI		FTA_{200}	
	+	−		−	+		+		+	−	+
1			1	1		15		9	6	9	6
1		1			1		4		4		4

Treated old syphilis shows non reactive FTA-200 and reactive TPI in steadily increasing degree the longer the interval from time of infection and the longer the period for which treatment has been given. Table 10 shows 8 cases of old treated syphilis in which FTA-200 is non reactive but FTA_{10} and FTA_{undil} are reactive

Table 11 comprises 113 persons in whom syphilis was suspected, 99 were reactive with both FTA-200 and TPI

Table 12 comprises 510 persons of whom not quite half were or are reactive with STS. Only three of these persons were found to be reactive with FTA-200 and TPI. Among 270 who were non reactive with STS there were two who were reactive with TPI and FTA-200

Table 12a gives the details of the serology of 19 persons 16 women and 3 men from the material of table 12 in order to elucidate in greater detail the specificity of the FTA-200 test. A number of the sera were very strongly reactive with STS but they were all non reactive with FTA-200 and TPI. Clinical diagnosis sex and age are recorded

At total of 1194 sera and 76 spinal fluid samples were examined the sera being from 1194 persons, spinal fluid samples from 33 of these, and from a further 43 persons, accounting for 1237 persons in all

All sera and spinal fluid samples had been sent to the Treponematoses Department for TPI testing, and throughout a certain period all samples sent in were also examined by the FTA test The sera and spinal fluids examined had thus been specially selected for TPI testing Many problems therefore, could be anticipated among the 1194 sera, such as would put the FTA test to a severe trial with regard to specificity and also to some degree with regard to sensitivity

TABLE 4

Primary Syphilis, 44 Untreated Cases

Disagreement between TPI and FTA$_{200}$						Agreement between TPI and FTA$_{200}$					
STS		TPI		FTA$_{200}$		STS		TPI		FTA$_{200}$	
−	+	−	+	−	+	−	+	−	+	−	+
1		1			1	10		7	3	7	3
	18	18			18		15	3	12	3	12

TABLE 5

Primary Syphilis, 64 Treated Cases

Disagreement between TPI and FTA$_{200}$						Agreement between TPI and FTA$_{200}$					
STS		TPI		FTA$_{200}$		STS		TPI		FTA$_{200}$	
−	+	−	+	−	+	−	+	−	+	−	+
						56		46	10	46	10
	1	1			1		7	2	5	2	5

RESULTS

Tables 4 and 5 cover 108 cases of primary syphilis, 44 untreated and 64 treated respectively As are most of those following, each of these tables is divided into two sections disagreement between TPI and FTA$_{200}$, and agreement between TPI and FTA$_{200}$, respectively

It is evident that FTA is more sensitive for primary, untreated syphilis than TPI

Tables 6 and 7 cover 62 cases of secondary syphilis, 41 untreated and 21 treated, respectively These 62 cases included only three cases giving different results with FTA$_{200}$ and TPI

Table 8 includes cases of late-symptomatic syphilis, cases of congenital syphilis, and finally 184 cases of non-specified stages of syphilis, but not including any primary or secondary cases Complete agreement is seen between FTA$_{200}$, TPI and STS for all the untreated cases, while

wards a better agreement between FTA_{undil} and TPI is seen in the cerebrospinal fluid, than between FTA_{200} and TPI in the serum The TPI test was done with 10 per cent serum and 10 per cent spinal fluid, respectively

TABLE 10

Results of Examinations of Sera from 8 Persons with Treated Old Syphilis Disagreement between TPI and FTA_{200} but Agreement between TPI, FTA_{10} and FTA_{undil}

STS		TPI		FTA			
	+	−	+	FTA_{200}	FTA_{10}	FTA_{undil}	
1			1		++	++++	Syphilis 1916
1			1	−	++++	++++	Syphilis 1925
1			1		++++	++++	Syphilis 1939
	1		1		not done	++++	Syphilis 1948
	1		1		not done	++++	Syphilis 1951
	1		1		++++	++++	Syphilis lat 1960
	1		1		++++	++++	Syphilis lat (From Sweden, no exact information)
	1		1		++	+++	Syphilis lat (From Rumania no exact information)

TABLE 11

113 Persons for which History Clinical and Serological Examination Support Suspicion for Syphilis—The Main Part Treated

Agreement between TPI and FTA_{200}

STS		TPI		FTA_{200}	
−	+	−	+		+
16		6	10	6	10
	97	8	89	8	89

TABLE 12

Agreement between TPI and FTA_{200}

STS		TPI		FTA_{200}	
		−	−	−	+
270		268	2	268	2
	240	237	3	237	3

TABLE 8

Results of Examinations of Sera from 307 Cases with Various Stages of Syphilis except Primary and Secondary

Agreement between TPI and FTA_{200}

	Untreated						Treated					
	STS		TPI		FTA_{200}		STS		TPI		FTA_{200}	
	−	+	−	+	−	+	−	+	−	+	−	+
Syphilis of skin mucous-membrane or bone		2		2		2		7		7		7
							3		1	2	1	2
Mb cordis luica		6		6		6		15		15		15
							1			1		1
Cerebrospinal syphilis		5		5		5		16		16		16
							13		2	11	2	11
Tabes dorsalis		1		1		1		7		7		7
							6		1	5	1	5
Dementia paralytica		4		4		4		8		8		8
							3			3		3
Lues cong		2		2		2		16	1	15	1	15
							8		3	5	3	5
Non specified stages of syphilis		7		7		7		88	5	83	5	83
							89		46	43	46	43

TABLE 9

Results of Examinations of Sera from 35 Cases with Various non Specified Stages of Treated Syphilis Showing Disagreement between TPI and FTA_{200}

STS		TPI		FTA_{200}	
−	+		+	−	+
14			14	14	
	10		10	10	
6		6			6
	2	2			2
	2	2			2
1			1	1	

Table 12b gives serological details of a further 18 persons, 11 women and 7 men, not included in the material of Table 12. They are all TPI non-reactive, some are also STS non reactive. They might all have been FTA reactive if this test had been carried out with undiluted serum in all cases. In addition, 8 out of the 18 were reactive with FTA_{200}.

Table 13 shows the results of serological examination of sera and cerebrospinal fluid samples from 33 people, 27 of whom suffered from various forms of syphilis, while 6 had other diseases. A tendency to-

wards a better agreement between FTA_{undil} and TPI is seen in the cerebrospinal fluid, than between FTA_{200} and TPI in the serum The TPI test was done with 10 per cent serum and 10 per cent spinal fluid, respectively

TABLE 10

Results of Examinations of Sera from 8 Persons with Treated Old Syphilis Disagreement between TPI and FTA_{200} but Agreement between TPI, FTA_{10} and FTA undil

STS		TPI		FTA			
	+	−	+	FTA_{200}	FTA_{10}	FTA_{undil}	
1			1		++	++++	Syphilis 1916
1			1	−	++++	++++	Syphilis 1925
1			1		++++	++++	Syphilis 1939
	1		1		not done	++++	Syphilis 1948
	1		1		not done	++++	Syphilis 1951
	1		1		++++	++++	Syphilis lat 1960
	1		1		++++	++++	Syphilis lat (From Sweden, no exact information)
	1		1		++	+++	Syphilis lat (From Rumania no exact information)

TABLE 11

113 Persons for which History Clinical and Serological Examination Support Suspicion for Syphilis—The Main Part Treated

Agreement between TPI and FTA_{200}

STS		TPI		FTA_{200}	
−	+	−	+	−	+
16		6	10	6	10
	97	8	89	8	89

TABLE 12

510 P ... xamination

Agreement between TPI and FTA_{200}

STS		TPI		FTA_{200}	
		—		−	+
270		268	2	268	2
	240	237	3	237	3

TABLE 12 a

From Table 12 are below given some details for 16 women and 3 men All STS reactive TPI and FTA$_{200}$ non-reactive Clinical diagnosis, sex and age are given

STS		Sex	Age in years	Clinical diagnosis
13/8	++	♀	59	Cancer colli uteri Spondylosis Myxoedema
8/4	++	♀	46	Metrorrhagia
1/1	±	♀	66	Anaemia haemolytica
5/2	+	♀	74	Tumor reg temporalis
13/9	++	♀	72	Leucosis myeloides
7/5	++	♂	67	Bronchitis chron
4/1	±	♀	82	Occl coron Febrilia
5/4	±	♀	48	Diab mellitus
14/4	±	♀	65	Lupus erythematosus
1/2	+	♂	50	Hemicrania Arteritis a. temporalis
4/5	++	♀	55	Anaemia perniciosa
11/10	++	♂	55	Cerebral symptoms
12/6	++	♀	75	Cholelithiasis Ulcus ventriculi
5/6	++	♀	18	Anaemia haemolytica
5/2	±	♀	60	Mb cordis rheum
5/1	≧+	♀	52	Diab mellitus
4/6	≧+	♀	69	Crises gastriques
2/4	+	♀	61	Arteriosol cerebri
1/3	+	♀	73	Ulcus ventriculi

TABLE 12 b

Besides details are given for 18 persons 11 women and 4 men not included in Table 12 and 12a Results of examination of sera in STS TPI and FTA$_{200}$ FTA$_{10}$ FTA$_{undil}$ Sex age and clinical diagnosis are given

STS		TPI	FTA			Sex	Age in years	Clinical diagnosis
			1 200	1 10	Undil			
/4	±		—		++++	♀	26	Pregnancy m IV
8/3	+			+	+++	♀	65	Mb Cushing
3/				++++	++++	♀	16	Mononucleosis inf
/8			+			♂	69	Bronchopneumonia
5/6	+		+	++	++	♀	79	Fractura colli femoris
			+	+++	++	♀	41	Epilepsia cryptogen
7/			+	++	++	♂	70	Tumor reg frontalis sin
/1			+	++	++	♀	60	Tumor renis
/			+	+++	++++	♀	55	Psychosis alcoholismus Dementia organ
/			+	+++	+	♂	68	Polyarthroitis
/6	—		++			♀	60	Bronchitis Otitis
–/1			++			♂	80	Aortaaneurism No information on syph
1/	—		++			♀	24	Gonorrhoea
7/–	—		++	+++	++	♂	75	Bronchitis Arthroitis
/–			++	++++	++++	♀	78	Adipositas Arthroitis
1/		—	++	++++	++	♂	31	Blood donor
–/–	—	—	++++			♀	58	Blood donor
/		—	++++			♂	41	Blood donor

Finally, Table 14 gives the results of a study of cerebrospinal fluid from 43 persons FTA_{undil} appears more reactive than TPI However, the number of examinations is small

Overall the 1194 sera examined showed 93 per cent agreement between FTA_{200} and TPI

TABLE 13

Results of the Examinations of Serum and Cerebrospinal Fluid from 33 Persons 27 Suffering from Various Forms of Syphilis and 6 Suffering from other Diseases Giving some Nervous Symptoms Justifying the Examination of Cerebrospinal Fluid

	Serum						Cerebrospinal fluid					
	STS		TPI		FTA_{200}		STS		TPI		FTA_{undil}	
		+	−	+	−	+	−	+	−	+	−	+
Tabes dorsalis	3			3	2	1	3		1	2	2	1
		1		1	1		1			1	1	
Dementia paralytica	1			1	1		1		1			1
		2		2		2	2			2		2
Cerebrospinal syphilis		6		6		6	3	3	1	5	3	3
Observatio pro	2			2	1	1	2		2		2	
Cerebrospinal syphilis		2		2	1	1	2		2		2	
Old treated syphilis with	4		3	1	3	1	4		4		4	
No cerebrospinal symptoms		5		5	3	2	5		5		5	
Primary syphilis untreated	1			1	1		1		1		1	
Other diseases than syphilis	4		4		4		4		4		4	
		2	2		2		2		2		2	

TABLE 14

Examination of Cerebrospinal Fluid from 43 Persons—No Examination of Serum

STS		TPI		FTA_{undil}		Clinical informations
−	+	−	+	−	+	
	1		1		1	All 7 persons with proved syphilis show some nervous symptoms
2			2		2	
4		4		1	3	
5		5		3	2	None of the 15 persons with proved syphilis show clinical symptoms of cerebrospinal syph
10		10		10		
21		21		21		History and clinical symptoms give no support for syphilis The examination of the cerebro spinal fluid indicated by *some nervous symp toms*

DISCUSSION

Nichols' strain of pathogenic Treponema pallidum is usually employed as antigen, although *Deacon et al* (6), *Pillot & Borel* (17), Co-

vert et al (3) and *Poetschke & Killisch* (18) tried to use Reiter's treponemes (see below)

The treponemes can be employed immediately the suspensions are prepared, and the suspensions can be used for periods of from weeks to months, so long as they are stored at 4° C Lyophilized treponemes can also be used, but the authors' observations suggest that the freshly isolated treponemes give the clearest microscopic pictures As indicated in Table 3, it is generally agreed that the antigen(the suspension of treponemes) should be air-dried on the slide A few workers then flame the slide lightly, but more use acetone fixation This is recommended by *Deacon* (4), as a clearer background is obtained, *i e* a cleaner microscopic picture *Borel & Durel* (1) are unable to confirm this, and *Nicl & Fribourg Blanc* (15) report that acetone treatment has an undesirable effect, the preparations giving a less clear microscopic picture The acetone can presumably be replaced by other fluids, *e g* ethanol or methanol (as used for example by *Poetschke & Killisch* (18)) In the present authors' laboratory, methanol has given excellent results It is possible that simple air drying is sufficient (15) The essential point seems to be that the treponemes must undergo special preparation in order to be useful as antigen in the FTA test

Thus, if the suspension of treponemes is mixed with syphilitic serum and untreated guinea-pig complement, as *e g* in the TPI test, the mixture incubated for 18 hours, and conjugate *then* added in an amount which will give a final conjugate dilution equal to that used in the ordinary FTA test, *no* fluorescence will be seen when the preparation is examined in the microscope On the other hand, if a specimen of the incubated mixture is placed on a slide air dried, acetone treated, and *then* treated with conjugate, an excellent fluorescence will appear The immobilized and killed treponemes have bound antibody, but cannot be made to fluoresce, before they have been subjected to further treatment It has never been possible to get mobile treponemes to fluoresce, although this should be possible, as during the course of the immobilization test there is an interval of up to 20 30 minutes in which up to half of the treponemes are sensitized although still not immobilized (experiments not yet published) The fluorescence of the immobilized treponemes described above can be elicited unchanged, even though the mixture of treponemes, antibody and complement is washed several times in saline to free the treponemes from complement and any possible antibody and other fixed or adsorbed substances that might be present This implies that the bound antibody is not released by this treatment on the other hand it does not necessarily imply that the antibody which in interaction with complement has elicited immobilization, is the same as the one responsible for the appearance of fluorescence, when interacting with conjugate

These experiments, together with much other experimental evidence, (*e g* lysozyme) (12), suggest that the surface of the treponeme is a

very important part of the organism, and worthy of considerable interest

Before use, it is customary to heat the serum to be examined in the FTA test to 56° C for 30 minutes (inactivation) With repeat examinations, it is further advised to reinactivate the sera for 10 minutes at 56° C, for each subsequent examination It is possible that this treatment is unnecessary or even undesirable *Nel & Fribourg Blanc* (15) report that they have stopped using this heat treatment, as in their opinion non specific results are provoked by the heating In contrast to this, *Grospiron* (9) considers that heat treatment at 62° C for 30 minutes can help to arrive at more specific results, although the treatment should preferably be combined with an absorption of the sera with powdered liver Grospiron's view agrees with *Smith's* observations (21) True treponemal antibodies show a greater thermostability than antilipoidal antibodies The present authors observed that adding varying amounts of untreated guinea pig serum to the serum to be examined in the FTA test, had no influence on the results

There is general agreement that fluorescein isothiocyanate is better than fluorescein isocyanate, in agreement with the findings of *Marshall et al* (11) It is uncertain whether an absorption of conjugate is of any real value *Grospiron* (9) reports that it is valueless to carry out an absorption treatment of conjugate, whereas an absorption of sera is useful, as indicated above It is possible that this state of affairs differs for the various preparations of conjugate, at any rate provided rabbits are used to produce the anti human gamma globulin

Deacon (5) recommends the addition of Tween 80 in 2 per cent solution as this substance should ensure better contact between antigen and antibody, and thereby ensure that when antibody is present in the sera being examined, fixation of antibody to antigen really does occur *Nel & Fribourg Blanc* question the value of Tweens, they believe that these actually reduced the binding, but suggest alternatively that it might have some significance in cleaning the microscopic preparation, so that it becomes clearer The present authors find that Tween is of doubtful value, but satisfactory studies to decide this have not been made nor does the literature provide information adequate enough to settle this question definitely

Deacon et al s paper (4) gives a comparison between the results obtained by a technique both with and without rotation of the preparations during treatment with serum and conjugate It is clear that by using rotation, 160 r p m with diameter approx 2 cm during the 2 × 30 minutes the sensitivity of the test is increased to a certain degree, as sera from two rabbits infected with Nichols' treponemes became reactive in FTA with rotation, 42 and 14 days after the inoculation, while without rotation the sera became reactive 64 and 49 days after the inoculation In a third rabbit, FTA with rotation became reactive 49 days after inoculation, while without rotation it was still non reactive after

112 days In contrast to this, *Borel & Durel* (1) and *Grospiron* (9) state summarily that rotation does not alter the sensitivity In the experience of the present authors, rotation as carried out by Deacon gives a very slight increase in sensitivity, but this is found to be so slight that it is insignificant (see below in the discussion on sensitivity)

The duration of treatment of the antigen spot with serum and later with conjugate has been determined by Deacon as 30 minutes in each case *Wilkinson* (26) uses 60 minutes, and *Grospiron* (9) has shown that while in most sera the maximum fluorescence is achieved after 60 minutes, the increase achieved after 30 minutes is negligible The conclusion is therefore, in acordance with Deacon's findings, that 30 minutes is a suitable interval both for sensitization with serum and for treatment with conjugate

The test was originally performed at room temperature, but further studies by *Deacon* (22), *Grospiron* (9) and others showed that it was more sensitive at 37° C, which should therefore be the preferred temperature

In the above, there has been repeated mention of circumstances enhancing the sensitivity of the FTA test, such as 1) rotating the slide, both during the serum treatment and during the subsequent treatment with conjugate, 2) extending the serum treatment beyond 30 minutes and finally, 3) performing the test at 37° C instead of at room temperature What would seem to be decisive in this connection, however, is the question of the reproducibility and specificity of the test From the very first experiments, it has in fact been observed that the test involves a non-specific element, and that it was necessary to dilute the serum examined in order to avoid non-specific results In view of this, it appears illogical to increase sensitivity by rotation, prolonged sensitization time and increased temperature, thereafter to reduce the sensitivity by diluting the serum Table 2 of *Deacon's* publication (5) shows that the reproducibility in experiments carried out with serum dilution 1 200 is better than when the serum dilution is 1 5, and this is an argument in favour of using the greater dilution If, thereafter, it is found necessary to increase the sensitivity, this can be done by employing the described modifications of the original technique Several authors claim that the specificity of the FTA test is at least as good, if not better than that of the TPI test (8) The present authors are unable to subscribe to this claim If, for example table 12b is examined, a number of persons are seen to have an FTA reactive serum, while TPI was non-reactive The information obtained about these cases definitely points to the FTA results as being non-specific It might be recalled in this connection that it is possible to use Reiter s treponemes in an FTA test and obtain approximately the same results as with the use of Nichols' treponemes (3, 17, 18) On the other hand, Reiter's treponemes can definitely not be employed in the TPI test, even the strongest TPI- and STS positive human syphilitic sera cannot immobilize Reiter s treponemes

The results given in table 12a are in agreement with those of *Pillot & Borel* (17), as these authors state that human sera reactive with STS alone, are non-reactive with FTA On the other hand, the results given in table 12b are at variance with those of *Pillot & Borel*

Provided the FTA test is carried out with sera in a primary dilution of 1 200, a specificity is obtained which lies close to that of the TPI test To described such an FTA test as being more sensitive than the TPI test is in a way misleading, as this great sensitivity can be claimed only by ignoring the necessity of diluting to 1 200 in order to obtain the required specificity It would appear more correct to put the matter as follows A) FTA_{200} is of somewhat lower specificity than the TPI test, and somewhat less sensitive, apart from primary syphilis (cf Table V (26)), or B) FTA_{undil} is considerably less specific than TPI, but considerably more sensitive In contrast to the TPI test, it appears *that the FTA test to a high degree involves a balance between sensitivity* and specificity, such as is familiar in tests using lipid antigens

Several authors have discussed whether immobilisin is identical with the antibody or antibodies which sensitizes the treponemes with respect *to conjugate A few have tended to show strong support in favour of* identity (8) *Borel & Durel* (1) have remarked that the number of antibody molecules required for a reactive FTA may be less than the number required for a reactive TPI, but that the antibody even so could be the same This explanation does not seem to harmonize well with the following arrangement of table 2b (from *Borel & Durel* (1)) for untreated secondary syphilis

Kline	Units	TPI titre	Average	FTA titre	Average
4	4	50	200	810	810
4		800		810	
8	8	10	126	2430	3027
8		100		7290	
8		200		810	
8		200		810	
8		800		21870	
16	16	10	86	810	5852
16		10		2430	
16		150		7290	
16		400		65610	
16		800		7290	
32	32	100	313	65610	16620
32		150		2430	
32		400		65610	
32		1600		7290	

It will be noted that Kline and FTA show a considerable correlation, while FTA and TPI titres show no correlation (cf (13) and (24))

It must be admitted that technically the FTA is relatively simple, and

the technique can presumably be simplified still further without any harm; for example, inactivation of sera and rotation could be omitted, and the test could be performed at room temperature provided this can be kept relatively constant. The specificity is satisfactory only if the sera are diluted primarily, and a dilution of 1:200 seems to be an excellent value. The reproducibility is surprisingly good. A quantitative FTA test seems to be considerably more attractive than a quantitative TPI test. In the International FTA study I, this laboratory obtained exceedingly satisfactory reproducibility and sensitivity, even though neither rotation nor a temperature of 37° C was used (25).

However, the TPI test still stands as the most specific test for syphilis and other treponematoses. We must hope that one in the future will prepare an antigen, presumably of treponemal origin, and in that case from the pathogenic treponemes, which has such specificity and sensitivity that a simple microreaction can replace TPI and FTA, and possibly the reagin reactions. Recent studies point the way to a polysaccharide antigen as a possible bearer of such characteristics. The polysaccharide from Reiter treponemes does not react with human syphilitic sera. In this connection it is tempting once more to mention that when Reiter treponemes are tried in an immobilization test with human syphilitic sera, they do not immobilize. Reiter polysaccharied reacts strongly in a complement fixation test with sera from rabbits immunized with Reiter treponemes. The presumed polysaccharide believed to have been isolated from Nichols' treponemes reacts in a complement fixation reaction with human syphilitic sera, but not with the above mentioned rabbit immune sera (2).

SUMMARY

On the basis of the available publications, a review is given of the results obtained in the FTA test, and a brief review is also given of the technique used by the different laboratories.

An investigation of 1191 sera and 76 cerebrospinal fluid samples from a total of 1237 persons is then reported. The methods used are described, and the results obtained tabulated. The sera used originate from persons with syphilis and from persons with other diseases.

The sensitivity, specificity and reproducibility of the FTA test are discussed, and its technique is examined in detail.

The conclusion is that the FTA method is of considerable interest for research work, and may possible be of use for diagnostic studies in laboratories where for various reasons the TPI test is difficult to carry out.

REFERENCES

1. *Borel, L. J. & Durel, P.* L'immunofluorescence appliquées au diagnostic de la syphilis. Path. Biol. 7: 2317, 1959.
2. *Christiansen, A. Hein.* Studies on the antigenic structure of Treponema pallidum. 3. Antigenic properties of polysaccharides isolated from Reiter's apathogenic strain. WHO/VDT/RES/16, 1962.

3 *Covert Scott V Kent John F & Stevens Roy W* Fluorescent treponemal antibody test using the Reiter treponeme Proc Soc exp biol med *106* 729, 1961
4 *Deacon W F Falcone V H & Harris Ad* A fluorescent test for treponemal antibodies Proc Soc exp biol med *96* 477, 1957
5 *Deacon W F Freeman F M & Harris Ad* Fluorescent treponemal antibody test Modification based on quantitation (FTA 200) Proc Soc exp biol med *103* 827 1960
6 *Deacon W F & Freeman F M* Fluorescent treponemal antibody studies J invest Derm *34* 249 1960
7 *Delacretaz J & Frenk E* Sensibilité et spécificité des tests treponémiques Dermatologica *122* 206 1961
8 *Fife Earl H Jr Bryan B M Sanders R W & Muschel L H* Evaluation of the fluorescent treponemal antibody (FTA) test for syphilis Comparison with Treponema pallidum immobilization (TPI) test Am J clin path *36* 105 1961
9 Grospiron Denise Étude des techn ... diagnos ... Faculté
10 *Harris Ad D* ... real Dis
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14 *Nairn R C* Fluorescent protein tracing E & S Livingstone Ltd Edinburgh and London 1962
15 *Nel G & Fribourg Blanc A* Technique actuelle du test d immunifluorescence appliqué au diagnostic de la syphilis Annales de l Institut Pasteur *102* 616 1962
16 *Nielsen H Aage* Investigations on the reproducibility of the quantitative Treponema pallidum immobilization test Acta path et microbiol scandinav *40* 119 1957
17 *Pillot J & Borel L-J* Étude des anticorps responsables de la ...
18 *Poets* ...
... 1959
19 Public Health Service Serology Evaluation and Research Assembly U S Department of Health Education and Welfare Bureau of State Services—Communicable Disease Center Atlanta Georgia 1956–1957
20 *Schmidt H* Cardiolipin antigen I A qualitative examination of sensitivity and specificity Brit J vener Dis 27 23 1951
21 *Schmidt H* The stability of anti lipoidal antibodies to heat inactivation at 56° C. Acta path et microbiol scandinav *51* 297 1961
22 Serology Tests for Syphilis Manual P H S Publ No 411 Washington 25 D C. 1959
23 *Thivolet J Grospiron D & Murat M* Utilisation de l immuno fluorescence pour le diagnostic de la syphilis (note préliminaire) Revue d hyg méd soc *8* 501 1960
24 *Vaisman A & Hamelin A* Application au diagnostic sérologique de la syphilis de la methode d immun fluorescence La Presse Med *69* 1157 1961
25 WHO internat FTA study I Department of Health Education and Welfare Public Health Service Communicable Disease Center Atlanta 22 Georgia
26 *Wilkinson A E* Fluorescent treponemal antibody test A preliminary report. Brit J vener Dis 37 59 1961

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On the basis of the available publications, a review is given of the results obtained in the FTA test, and a brief review is also given of the technique used by the different laboratories.

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REFERENCES

1. *Borel, L. J. & Durel, P.*: L'immunofluorescence appliquées au diagnostic de la syphilis. Path. Biol. 7: 2317, 1959.
2. *Christiansen, A. Hein*: Studies on the antigenic structure of Treponema pallidum. 3. Antigenic properties of polysaccharides isolated from Reiter's apathogenic strain. WHO/VDT/RES/16, 1962.

infected with SV_{40} or left as controls $10^{5.5}$ $TCID_{50}$ of a strain that could be neutralized by antiserum to strain 776 was added (The virus was kindly supplied by Dr D I Magrath Research Council Laboratories, London) It had had several passages in this laboratory in cultures of kidney cells from the African green monkey The titrations were performed in such cultures in roller tubes

The coverslips were taken from the plates at intervals of 4 hours and 1, 2 3 5 8, 11 and 14 days after the infection They were rinsed in phosphate buffered saline at pH 7 3 dried in air, and fixed for 10 minutes in acetone

The indirect fluorescent antibody technique (*Weller & Coons* 1954) was used *Anti SV_{40} serum was obtained from a rabbit inoculated three times with a virus*

This procedure was carried out twice For the first adsorption 300 mg powder per ml was used for the second 100 mg per ml

Two drops of the anti SV_{40} serum were placed on the coverslips which were incubated in a humidity chamber at 37° for 60 minutes They were then rinsed several times in phosphate buffered saline and 2 drops of the anti rabbit globulin solution were added After incubation for 60 minutes they were rinsed in saline and mounted in phosphate buffered glycerine on slides Some of the coverslips with infected cells were treated with rabbit antiserum to polyoma virus instead of the anti SV_{40} serum before the application of the anti rabbit globulin

RESULTS

In all the non-infected cultures, in the infected cultures fixed 4 and 24 hours after infection, and in the infected cultures treated with antiserum to polyoma virus only a faint fluorescence, predominantly in the cytoplasm, was found (Fig 1)

A specific antibody staining was seen in the cultures after 2 days and later after infection In the 2-day-specimens a few scattered nuclei presented a faint granular fluorescence (Fig 2) On the 3rd day after infection the number of the fluorescent nuclei as well as the intensity of the fluorescence had increased somewhat Five days after infection about 50 per cent of the cells were affected The fluorescence was seen in the nuclei and its intensity had increased considerably (Fig 3) The number of nuclei with a diffuse fluorescent staining was higher than the number of nuclei with a granular staining Eight days after infection a specific cytoplasmic fluorescence appeared in a few cells It was granular and rather faint At the later stages of infection it was more diffuse and intense, while the nuclear fluorescence decreased greatly or disappeared In the 14-day-specimens about 70 per cent of the cells had specific fluorescent material, most of which was found in the cytoplasm (Fig 4)

The virus caused cytopathic changes in the cultures Many nuclei swelled to several times their normal volume This was observed already on the 2nd day after infection In the phase microscope a cytoplasmic vacuolation was noted in a few cells 5 days after infection The number of these cells increased gradually and in the 14-day specimens

FROM THE INSTITUTE OF VIROLOGY, UNIVERSITY OF UPPSALA
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A FLUORESCENT ANTIBODY STUDY ON THE FORMATION OF SIMIAN VIRUS 40 IN MONKEY KIDNEY CELLS

By

HANS DIDERHOLM

Received 6 x 62

The simian virus 40 (SV_{40}) is present in many kidney cell cultures of rhesus and cynomolgus monkeys (*Sweet & Hilleman* 1960) The agent grows but does not cause any obvious cytopathic effect in its host cultures Instead, it grows to high titres with a marked cytopathic effect in kidney cell cultures of the African green monkey, *Cercopithecus aethiops* The virus has also been named vacuolating agent because of the characteristic cytoplasmic vacuolation seen in cells in infected cultures

The presence of SV_{40} in monkey kidney cultures constitutes a problem in the preparation of polio and adenovirus vaccines The virus has been isolated from all three types of Sabin's vaccine (*Sweet & Hilleman* 1960) It is relatively resistant to inactivation by formaldehyde, and it has been shown that certain lots of formaldehyde inactivated polio and adenovirus vaccines contain infectious SV_{40} virus (*Gerber et al* 1961) Attention has been drawn to the question whether the virus might cause adverse effects, but, at present there is no evidence of SV_{40} being harmful in man In hamsters, however, SV_{40} is able to induce sarcomas visible about 6 months after infection (*Girardi et al* 1962 and *Eddy et al* 1962)

The present report deals with the formation of SV_{40} in kidney cell cultures of the African green monkey The indirect fluorescent antibody technique described by *Weller & Coons* (1954) is used

MATERIAL AND METHODS

Cultures of trypsinized kidney from the African green monkey *Cercopithecus aethiops*, were set up in flasks with 94 5 per cent Hanks' solution 0 5 per cent lactalbumin hydrolysate and 5 per cent heat inactivated calf serum After 5 days subcultures were made The cells were seeded into 60 mm plastic petri plates which contained a sterile coverslip Each plate received about 3×10^6 cells in 5 ml of Parker's solution with 2 per cent heat inactivated calf serum All culture media contained 100 units of penicillin and 100 μg of streptomycin per ml

When monolayers had been formed the cultures were washed refed and either

This investigation was supported by grants from the *Swedish Cancer Society*



Fig 3
Five days after infection with SV₄ virus The specific nuclear fluorescence is intense and diffuse rather than granular (× 350)

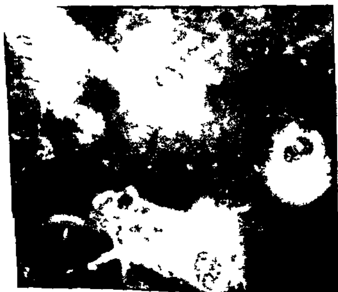


Fig 4
Fourteen days after infection with SV_4 virus Most of the specific fluorescence is seen in the cytoplasm (× 350)



Fig. 1
Non infected cells. A faint autofluorescence is seen (× 350)



Fig. 2
Two days after infection with SV4 virus. Specific granular fluorescence is seen in the nuclei (× 350)



Fig 3

Five days after infection with SV_{41} virus The specific nuclear fluorescence is intense and diffuse rather than granular (× 350)

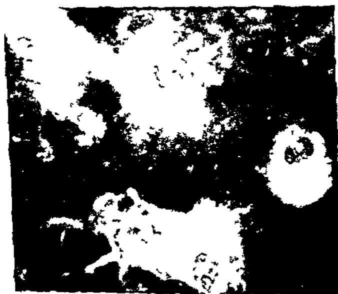


Fig 4

Fourteen days after infection with SV_{41} virus Most of the specific fluorescence is seen in the cytoplasm (× 350)

most of the cells showed vacuolation At this stage of infection also other cytopathic changes were obvious Still a certain number of cells presented normal aspects but a fluorescent nucleus It is not possible to decide whether these cells had a latent infection or represented newly infected cells

DISCUSSION

The results indicate that viral antigen is first formed in the nuclei of the cells subsequently to migrate into the cytoplasm The results do not exclude that viral antigen may also be formed in the cytoplasm, where it may cause part of the specific fluorescence visible at later stages of infection

The results are on the whole in agreement with the results obtained by *Henle et al* (1959) in studies on polyoma virus in mouse embryo cultures At earlier stages of infection they noted a specific fluorescence in the nuclei At later stages specific fluorescence appeared in the cytoplasm, while the nuclei lost their fluorescent staining Similar results were also obtained with herpes simplex virus (*Lebrun* 1956), and also the g-antigen of the fowl plague virus seems to be formed in the nucleus before it migrates into the cytoplasm (*Breitenfeld & Schafer* 1957)

SUMMARY

The indirect fluorescent antibody technique was used to study the formation of simian virus 40 (SV_{40}) in kidney cell cultures of the African green monkey It was found that a few nuclei contained specific fluorescent material 2 days after infection During the next 3 days more nuclei were affected and the intensity of the fluorescence increased Thereafter a specific cytoplasmic fluorescence appeared, which increased with time, while the nuclear fluorescence decreased or disappeared

REFERENCES

Breitenfeld P M & Schafer W The Formation of Fowl Plague Virus Antigens in Infected Cells as Studied with Fluorescent Antibodies Virology 4 328 345 1957

Eddy B E Borman G S Grubbs G F & Young R D Identification of the Oncogenic Substance in Rhesus Monkey Kidney Cell Cultures as Simian Virus 40 Virology *17* 65 75 1962

Gerber P Hottle G A & Grubbs R F Inactivation of Vacuolating Virus (SV_{40}) by Formaldehyde Proc Soc Exptl Biol Med *108* 205 209 1961

Girardi A J Sweet B H Slotnick V B & Hilleman M R Development of Tumors in Hamsters Inoculated in the Neonatal Period with Vacuolating Virus SV_{40} Proc Soc Exptl Biol Med *109* 649 660 1962

Henle G Deinhardt F & Rodriguez J The Development of Polyoma Virus in Mouse Embryo Cells as Revealed by Fluorescent Antibody Staining Virology 8 388 391 1959

Lebrun J Cellular Localization of Herpes Simplex Virus by Means of Fluorescent Antibody Virology 2 496 510 1956

Sweet B H & Hilleman M R The Vacuolating Virus SV_{40} Proc Soc Exptl Biol Med *105* 420–427, 1960

Weller T H & Coons A H Fluorescent Antibody Studies with Agents of Varicella and Herpes Zoster Propagated *in vitro* Proc Soc Exptl Biol Med *86* 789 794 1954

FROM THE DEPARTMENT OF PATHOLOGY POSTGRADUATE MEDICAL SCHOOL OF LONDON

OBSERVATIONS ON THE EFFECT OF POTASSIUM FERRI AND FERROCYANIDES ON THE FORMATION OF INDIGO FROM INDOXYL ACETATE SPLIT BY ALKALI AND LIVER HOMOGENATES[1]

By

JOHAN AHLQVIST

Received 11 ix 62

When some halogen substituted indoxyl acetates are used for the histochemical demonstration of non specific esterases the use of low concentrations of potassium ferri and ferrocyanides (oxidizer) gives a diffuse localization of the indigos in liver cells and cells in the tubules of the kidney cortex in rats With high concentrations the deposition of indigos is restricted to peribiliary granules in the liver and intracytoplasmatic droplets in the kidney (*Holt & Withers* 1952, *Holt & Withers* 1958 *Shnitka & Seligman* 1961) *Holt* (1956) was of the opinion that the main effect of the oxidizer was to accelerate the conversion of liberated indoxyl to indigo *Pearse* (1960) suggested that differential inhibition of esterases or loss of lyo-esterase might be involved Pearse also points out that absolute quantitation of enzyme activity is difficult since according to *Holt* (1958) the ratio between indigo and colourless by products decreases with increasing pH *Shnitka & Seligman* (1961) regard the more precise localization of indigo in the presence of high concentration of the oxidizer to be due to inhibition of a sensitive esterase by the oxidizer On this matter differences of opinion thus still seem to exist It was thought worth while to investigate the matter further

Solutions of indoxyl acetate were made up with different concentrations of ferri and ferrocyanides (oxidizer) The solubility of the indoxyl acetate was not noticeably influenced by the oxidizer as shown by the separation of the ester from the oxidizer-containing solutions and the hydrolysis of it with NaOH in the absense of oxidizer leading to the formation of blue indigo Very little blue indigo formed after hydrolysis of the ester with NaOH in the presence of high concentrations of oxidizer although the hydrolysis itself was not influenced by the oxid

[1] The author was a British Council Scholar

most of the cells showed vacuolation At this stage of infection also other cytopathic changes were obvious Still a certain number of cells presented normal aspects but a fluorescent nucleus It is not possible to decide whether these cells had a latent infection or represented newly infected cells

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The results indicate that viral antigen is first formed in the nuclei of the cells subsequently to migrate into the cytoplasm The results do not exclude that viral antigen may also be formed in the cytoplasm, where it may cause part of the specific fluorescence visible at later stages of infection

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Henle G Deinhardt F & Rodriguez J The Development of Polyoma Virus in Mouse Embryo Cells as Revealed by Fluorescent Antibody Staining Virology 8 388 391 1959

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Weller T H & Coons A H Fluorescent Antibody Studies with Agents of Varicella and Herpes Zoster Propagated *in vitro* Proc Soc Exptl Biol Med 86 789 794 1954



Fig. 1

Concentration of ferri- and ferrocyanides in the original solutions from left to right 10, 5, 3.75, 2.5, 1.25 and 0 mM respectively. 4.5 mg indoxyl acetate per 10 ml. The solutions were filtered before treated as below.

Top: To 5 ml of the above added 2.5 ml of N NaOH. The colour seems to be inversely related to the concentration of the oxidizer.
Bottom: The indoxyl acetate was extracted from the original solutions with chloroform and demonstrated with NaOH in the remainder after evaporation of the chloroform. The colour is approximately the same in all the tubes.

in the absence of oxidizer within a few seconds leads to the formation of a strong blue colour. The blue indigo soon flocculates. When ferri- and ferrocyanides are present in high concentrations the formation of the blue colour is considerably delayed and diminished. After *2* minutes the solutions mostly have a brownish colour; when shaken later the solution turns blue, the intensity of the colour depending on the concentration of the oxidizer (Fig. 1). Attempts to separate unsplit indoxyl acetate from the solutions after the addition of NaOH gave negative results in all instances. The indigo formed was first removed by filtration and the filtrate extracted with chloroform. The remainder after evaporation of the chloroform was dissolved; no blue colour developed when NaOH was added. This indicates that no unsplit indoxyl acetate remainded in spite of the fact that less blue indigo had formed under the influence of the oxidizer.

Since the *oxidizer did not affect the solubility of the indoxyl acetate* to a noticeable degree and since no unsplit ester remained in the solutions after hydrolysis with NaOH it seems obvious that under the influence of the oxidizer some compounds which were not blue must have been formed. These compounds at least in part are thought to make the solutions light brown. The tests agree with the findings of *Holt* (1958) that under alkaline conditions the presence of ferri- and ferrocyanides leads to the formation of uncoloured by-products. The solubility of the

izer It is therefore clear that by-products which were *not blue* must have been formed by the action of the oxidizer Comparable results were obtained when the ester was hydrolysed by a liver homogenate at pH 5 6 The results indicate that in histoenzymatic indigogenic staining methods the formation of such by-products *should be given more consideration* than hitherto

METHODS

The solutions or incubating media were slightly modified from the method given by *Holt & Withers* (1952) and consisted of 0 1 N *acetate buffer pH 5 6 2 ml* 0 1 M $CaCl_2$ 1 ml and distilled water to make 10 ml after the addition of the desired volumes of 0 05 M solutions of potassium ferri and ferrocyanides The latter were stored at 4° C and always used within three hours of preparation In the first tests on the solubility etc of indoxyl acetate the above solutions were poured onto 0 3 ml of a 1 5 per cent solution of indoxyl acetate in ethanol in the tests with liver homogenates 0 2 ml of a 2 per cent solution of the ester in ethanol was introduced through a submerged pipette

Under aerial conditions NaOH splits indoxyl acetate leading to the formation of coloured indigo (*Holt & Sadler* 1958) 1 N NaOH was employed in these tests To separate indoxyl acetate from a solution containing the oxidizer the solution was shaken with the same volume of chloroform for two minutes the chloroform separated and evaporated at 70° C The remainder was tested *for the presence of* indoxyl acetate by dissolving it in 0 5 ml ethanol and 3 ml of distilled water and adding 1 ml of N NaOH which leads to the formation of blue indigo in the presence of indoxyl acetate The time of extraction proved sufficient no indoxyl acetate could be detected with NaOH in the water phase of oxidizer free solutions of indoxyl acetate after 2 minutes shaking with chloroform

For the biochemical tests a liver homogenate was prepared by treating 8 g of rat liver and 40 ml of chilled saline on a Waring blender for 4 minutes After centrifugation for 4 minutes at about 3 000 rpm on a small laboratory centrifuge to remove larger particles the supernatant was stored at 4° C and used within 2 hours of preparation An inactive homogenate was prepared by *heating a small volume* of the fresh one in a small glass mortar kept in boiling water for 4 minutes It was thereafter rehomogenized by hand In the biochemical tests 0 1 ml of these homogenates were used per 10 ml of incubating medium Incubation was usually carried out at 37° C for 1½ hours + 15 minutes Indoxyl acetate was separated from these suspensions by diluting them with the same volume of distilled water thereafter they were either centrifuged at about 3 000 rpm for 10 minutes or filtered and the supernatant or filtrate shaken with the same volume of chloroform the chloroform separated and evaporated and the remainder treated as above

Further details of the procedure are given below

EXPERIMENTS

The effect of the oxidizer on the solubility of indoxyl acetate and on the formation of blue indigo from the ester after NaOH hydrolysis When incubating media with different concentrations of oxidizer, after filtration through Whatman filter paper No 1, were extracted with chloroform and indoxyl acetate demonstrated with NaOH in the remainder after evaporation of the chloroform, no differences between the samples could be detected, at least no such differences as might be responsible for the diminished formation of indigo under the influence of oxidizer as demonstrated below This point is shown in Fig 1

The addition of 5 ml of N NaOH to 10 ml of the incubation medium

in the table also 10 and 1mM concentrations were employed In this series the indigo formed was extracted from the whole incubation medium with a ten-fold volume of chloroform for colorimetry At colorimetry at 600 mμ the following readings (E × 100) were obtained 1 5 for the extract from the incubation medium with a 10 mM concentration of oxidizer, 4 5 for the one with a 5 mM concentration, 23 for the one with a 1 mM concentration, 21 5 for the one with a 0 5 mM concentration and 10 5 for the extract from the incubating medium with oxidizer omitted The control incubated with inactivated homogenate at a 5 mM concentration of the ferri- and ferrocyanides gave a reading of 0 or the same as the blank which consisted of pure chloroform The colorimetry thus confirmed results given in the table

The brownish yellow colours in the chloroform extracts from the filtrates or supernatants of the incubating media with a 5 mM concentration of the oxidizer are thought to be due to the same substances which gave these colours to the incubation media The violet colours in the extracts from the incubation media with oxidizer omitted or present at low concentration are presumably due to some intermediate

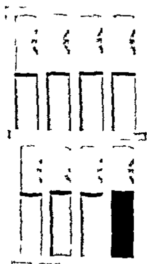


Fig 2

Concentration of ferri and ferrocyanides in the original incubation media from left to right 5 1 0 5 and 5 mM respectively In the last tube inactivated in the others fresh homogenate 3 mg indoxyl acetate per 10 ml

Top — Indigo extracted from 4 ml of whole incubation media with 40 ml of chloroform after incubation First tube brownish yellow with uncertain bluish tint 2nd and 3rd tubes blue, last one colourless

Bottom — Unsplit indoxyl acetate extracted from filtrates of remaining 6 ml of incubation media after incubation After separation the chloroform was evaporated and the remainder dissolved and treated with NaOH for demonstration of unsplit substrate First tube light brownish yellow, 2nd and 3rd ones light violet last one strongly blue

indoxyl acetate apparently is not affected by the oxidizer as was earlier suspected by the author.

Effect of oxidizer on formation of indigo from indoxyl acetate hydrolysed by liver homogenate at pH 5.6. The main results are tabulated. The colour of the incubation media with the same concentration of oxidizer at the end of incubation showed fairly small variations in the different tests. Inactivated homogenate as was expected, caused no changes in colour of the incubation medium. Active homogenate induced the strongest blue colours at low concentrations of oxidizer. In one case only out of 4 did a faint blue colour develop at the high 5 mM concentration of the oxidizer, in 3 cases light brownish-yellow colours developed. These clearly differed from the original yellow colour of the oxidizer and seemed similar to the ones developing from indoxyl acetate split by NaOH under the influence of the oxidizer. Once after recording the colours after 1½ hours incubation this was prolonged to 14 hours without any essential effect on the colour of the suspensions.

In one series of tests in addition to concentrations of oxidizer shown

TABLE 1

Effect of Different Concentrations of Ferri- and Ferrocyanides on Formation of Indigo from Indoxyl Acetate upon by Fresh (act.) and Heat Inactivated (inact.) Liver Homogenate

Concentration of ferri- and ferrocyanides and kind of homogenate	5 mM act.	0.5 mM act.	0 mM act.	0 mM inact.	5 mM inact.
No. of tests	4	4	4	4	1
Incubation medium at end of incubation	3 cases brownish yellow 1 brownish yellow with blue tint	blue with greenish tint	pale blue with greenish tint	colourless	pale yellow
Chloroform extract from filtrate or supernatant of incubation medium	brownish yellow	3 cases light violet 1 yellowish tint	light violet	colourless	colourless
Above evaporated remainder dissolved and NaOH added	2 cases brownish yellow 2 faint violet tint	3 cases light violet or blue, 1 light brownish yellow	3 cases light violet or blue 1 sample lost	strong blue	strong blue

After incubation the filtrate or supernatant of the incubation medium was chloroform extracted, the chloroform evaporated after separation and the remainder dissolved in ethanol and water and treated with NaOH under aerial conditions for the demonstration of unsplit indoxyl acetate. Details of the procedure in the text.

in the table also 10 and 1mM concentrations were employed In this series the indigo formed was extracted from the whole incubation medium with a ten-fold volume of chloroform for colorimetry At colorimetry at 600 mμ the following readings (E × 100) were obtained 1 5 for the extract from the incubation medium with a 10 mM concentration of oxidizer, 4 5 for the one with a 5 mM concentration, 23 for the one with a 1 mM concentration, 21 5 for the one with a 0 5 mM concentration and 10 5 for the extract from the incubating medium with oxidizer omitted The control incubated with inactivated homogenate at a 5 mM concentration of the ferri- and ferrocyanides gave a reading of 0 or the same as the blank which consisted of pure chloroform The colorimetry thus confirmed results given in the table

The brownish-yellow colours in the chloroform extracts from the filtrates or supernatants of the incubating media with a 5 mM concentration of the oxidizer are thought to be due to the same substances which gave these colours to the incubation media The violet colours in the extracts from the incubation media with oxidizer omitted or present at low concentration are presumably due to some intermediate



Fig 2

Concentration of ferri and ferrocyanides in the original incubation media from left to right 5 1 0 5 and 5 mM respectively In the last tube inactivated, in the others fresh homogenate 3 mg indoxyl acetate per 10 ml

Top Indigo extracted from [illegible] for after incubati[illegible] tint 2nd and 3rd [illegible]

Bottom Unsplit indoxyl ac[illegible] bation media after [illegible] porated and the re[illegible] [illegible] treated with NaOH for demonstration of unsplit substrate First tube light brownish yellow, 2nd and 3rd ones light violet last one strongly blue

compounds or by-products formed from liberated indoxyl; they never were clearly blue, they never occured in extracts from samples with inactive homogenate and the colour at least sometimes seemed to grow stronger in the chloroform before evaporation

After evaporation of the chloroform from extracts of filtered samples with oxidizer omitted or present at low concentration, the remainder when dissolved in ethanol and water mostly had a light violet colour Upon addition of NaOH for the demonstration of remaining unsplit indoxyl acetate the colour sometimes seemed to remain unchanged, sometimes it grew stronger turning bluer Solutions from the samples incubated with inactivated homogenate in a few seconds turned strongly blue when NaOH was added, in the same manner as do oxidizer-free solutions of indoxyl acetate The violet colours in the samples in which a high concentration of oxidizer had been employed always were very faint if present at all After evaporation of the chloroform only in the cases in which inactive homogenate had been used did a strong "moth-ball" smell occur This was present in all these cases and never occurred if active homogenate had been employed As far as can be judged it was identical to the smell of indoxyl acetate In one series after addition of NaOH the solutions were extracted with chloroform for colorimetry The extract from the solution in which inactivated homogenate and a 5 mM concentration of the oxidizer had been employed gave a reading of 37 5, extracts from the solutions in which active homogenate and 10, 5 and 0 5 mM concentrations of oxidizers were used gave readings of 1 5, 3 5 and 5 respectively

Examples of these tests are given in Fig 2

DISCUSSION

It seems obvious that faintly coloured or colourless substances must have formed from indoxyl liberated by NaOH under aerial conditions in the presence of high concentrations of ferri- and ferrocyanides This agrees with *Holt*'s (1958) statement that under alkaline conditions colourless by-products are formed from part of liberated indoxyl in the presence of ferri- and ferrocyanides In the in vitro tests with liver homogenate very little indigo formed in incubation media containing a high concentration of the oxidizer, in spite of the fact that an acid pH was employed Very little, if at all, unsplit indoxyl acetate remained in such incubation media This indicates that the decreased formation of blue indigo in the presence of high concentration of oxidizer in these tests also must be due to an increased formation of colourless or faintly coloured by-products and not to any significant inhibition of enzyme

It should be pointed out that the findings by no means constitute "new knowledge", the formation of colourless by-products has been clearly mentioned by *Holt* (1958) and the possible consequences of this seem to have been fully realized by *Pearse* (1960).

The formation of less indigo in the tests without oxidizer and with active homogenate than when low concentration of the oxidizer was used is not thought necessarily to indicate that the responsible enzymes were activated by the ferri and ferrocyanides. This effect might for instance have been due to a slower oxidation of liberated indoxyl at the pH employed (*Colson & Holt* 1958).

Although in the tests with liver homogenates the use of an acid pH might have made other enzymes than non specific esterases responsible for the splitting of the indoxyl acetate (*Hess & Pearse* 1958) it might be permissible to comment on the interpretations of the more precise localization of indigo in liver and in kidney tubules in reactions for esterases with halogen substituted indoxyl acetates and high concentrations of oxidizer. Holt (1956) regarded the main effect of the oxidizer to be to reduce diffusion of liberated indoxyl. Holt & Hobbiger (cited by *Holt* 1958) state that esterases are not inhibited by ferri and ferrocyanides. *Shnitka & Seligman* (1961) clearly seem to have shown that when such high concentrations of the oxidizer are used in indigogenic histochemical methods a large part of the esterases do not give deposition of dye. Their findings thus disagree with the opinion of *Holt* (1956). Their interpretation of this decreased deposition of indigo is that part of the esterases were inhibited by the ferri and ferrocyanide. They do not however offer direct evidence for this opinion since for technical reasons they could not incorporate ferri and ferrocyanides in their azo dye methods for esterases. In the light of the present investigation it therefore seems that before their theory can be finally accepted it should be excluded that an increased formation of colourless by products from liberated halogen substituted indoxyl under the influence of ferri and ferrocyanide is not responsible for this decreased formation of blue indigo.

The results indicate that in all histo enzymatic indigogenic staining reactions the formation of uncoloured or faintly coloured by products from liberated indoxyl should be considered in the interpretation of the results.

More reliable results might be obtained if liberated indoxyl in histo-enzymatic reactions is coupled with diazonium salts as employed by *Hess & Pearse* (1961 62).

SUMMARY

Potassium ferri and ferrocyanides in 5 mM concentrations induce the formation of colourless or faintly coloured by products from a large part of indoxyl liberated from indoxyl acetate by NaOH or at pH 6 by liver homogenates both under aerial conditions. The possible consequences of this in the interpretation of histo enzymatic indigogenic staining reactions are discussed.

REFERENCES

1 *Cotson S & Holt S J* Studies in enzyme histochemistry IV Kinetics of aerial oxidation of indoxyl and some of its halogen derivates Proc Roy Soc B *148* 506 519 1958
2 *Hess R & Pearse A G F* The histochemistry of indoxylesterase of rat kidney with special reference to its cathepsin like activity Brit J exp Path 39 292 299 1958
3 *Hess R & Pearse A G F* Enzyme immobilization Substantive diazonium salts in enzyme histochemistry Enzymol biol clin *1* 87 113 1961–62
4 *Holt S J* The value of fundamental studies of staining reactions in enzyme histochemistry with reference to indoxyl methods for esterases J Histochem Cytochem 4 541 502 1956
5 *Holt S J* Indigogenic staining methods for esterases In General Cytochemical Methods Ed J F Danielli Vol 1 pp 375 398 Academic Press Inc New York 1958
6 *Holt S J & Sadler P W* Studies in enzyme histochemistry III Relationship between solubility molecular association and structure in indigoid dyes Proc Roy Soc B *148* 495 505 1958
7 *Holt S J & Withers R F J* Cytochemical localisation of esterases using indoxyl derivates Nature Lond *170* 1012 1014 1952
8 *Holt S J & Withers R F J* Studies in enzyme histochemistry V An appraisal of indigogenic reactions for esterase localisation Proc Roy Soc B *148* 520 533 1958
9 *Pearse A G F* Histochemistry Theoretical and Applied 2nd edition Churchill London 1960
10 *Shnitka T K & Seligman A M* Role of esteratic inhibition on localisation of esterase and the simultaneous cytochemical demonstration of inhibitor sensitive and resistant enzyme species J Histochem Cytochem *9* 509 527 1961

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METASTATIC CARCINOMA TO THE JAWS

By

FINN CLAUSEN and HEMMING POULSEN

Received 10 x 62

Metastatic involvement of the mandible and maxilla is not uncommon as part of generalized metastasis from carcinomas developing at widely different sites, especially breast, bronchi and kidney.

Less frequent and more interesting is it that metastasis to the jaw bones may occur at an early stage of the disease and sometimes represent the first symptom of a metastatic carcinoma and of the primary tumour as well.

These cases should receive special consideration because case histories reported in the literature show that they are often overlooked or misinterpreted in many cases for several months. From a diagnostic point of view metastatic carcinoma of the jaws plays a role, both clinically and radiographically, in the differentiation from diseases such as, for example, cysts, bone abscesses, osteomyelitis, and primary tumours of the bone.

The aim of this work has been to call attention to the pathological picture of metastatic carcinomas involving the jaws, further to carry out an analysis of the case reports published in the literature as well as to present five own cases, and finally to analyze the symptomatology and pathological anatomy of metastatic carcinomas of the jaw bones.

MATERIAL

The investigation comprised a total of 200 cases of tumour tissue in the jaw bones [illegible] nation [illegible] five. T[illegible] criteria [illegible] necessary to establish.

First, the lesion must be a true metastasis localized to the bone tissue as distinguished from direct invasion by a primary tumour in relation to the jaws, and from metastases to the [illegible] cally verified carcinoma [illegible] known. The last require[illegible] dealing with a secondar[illegible]

The authors want to express their gratitude to professor *Erik Husted*, M.D., to pathologist-in-chief *William Kjær*, M.D., to *Jørgen Rud*, D.D.S., odontologist, and *Frederik Wagner*, D.D.S., for the permission to use material from their patients.

[1] A list of the literature reviewed will be sent upon request.

REFERENCES

1 *Cotson S* & *Holt S J* Studies in enzyme histochemistry IV Kinetics of aerial *oxidation of indoxyl and some of its halogen derivates* Proc Roy Soc B *148* 506 519 1958

2 *Hess R* & *Pearse A G E* The histochemistry of indoxylesterase of rat kidney with special reference to its cathepsin like activity Brit J exp Path 39 292 299 1958

3 *Hess R* & *Pearse A G E* Enzyme immobilization Substantive diazonium salts in enzyme histochemistry Enzymol biol clin *1* 87 113 1961 62

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5 *Holt S J* Indigogenic staining methods for esterases In General Cytochemical Methods Ed J F Danielli Vol 1 pp 375 395 Academic Press Inc New York 1958

6 *Holt S J* & *Sadler P W* Studies in enzyme histochemistry III Relationship between solubility molecular association and structure in indigoid dyes Proc Roy Soc B *148* 495 505 1958

7 *Holt S J* & *Withers R F J* Cytochemical localisation of esterases using indoxyl derivates Nature Lond *170* 1012 1014 1952

8 *Holt S J* & *Withers R F J* Studies in enzyme histochemistry V An appraisal of indigogenic reactions for esterase localisation Proc Roy Soc B *148* 520 533 1958

9 *Pearse A G F* Histochemistry Theoretical and Applied 2nd edition Churchill London 1960

10 *Shnitka T K* & *Seligman A M* Role of esteratic inhibition on localisation of esterase and the simultaneous cytochemical demonstration of inhibitor sensitive and resistant enzyme species J Histochem Cytochem 9 509 527 1961



1

2

Figs 1 and 2

Fig 1 Case No 1 Radiogram of the lower jaw of a 60 year-old man There is a marked re rption of bone between the lower right can ne and lateral incisor

Fig 2 Case No 1 Histological examination of the metastas s shows adenocarcinoma Haemalum eosin × 3?0

Simultane u ly with the appearance of the mandibular metastasis the patient felt pains in the lumbar region Instead of stilbestrol he was given ovex which relieved the pain In addition radiotherapy was administered to the jaw tumour which failed to decrease in size but became painless During the following year the tumour continued to grow quite slowly and from December 1937 pains in the jaw set in again Within the following months the patient s condition became gradually worse and he died on the 5th August 1938 Autopsy showed a prostatic carcinoma with widely disseminated bone metastases and carcinosis pleurae

RESULTS

Case Reports

Examination of own records revealed five cases which met the above mentioned requirements All five cases were found to be metastatic carcinomas of the mandible The case reports will be outlined below

Case 1

A 60 year old man consulted his physician on the 21th December 1956 for a hemispherical indolent swelling of the gingiva round 3 2 —1 which he had noted two weeks previously There was retraction of gingiva and the radiograph showed a horizontal bone loss of about 5 mm between the two teeth (Fig 1) There was no fracture Thirteen days later 3 2 — were extracted revealing the presence of foul smelling pus in the alveolus The wound healed normally during the following three weeks but as the swelling failed to subside the gingival tumour was extirpated on the diagnosis of epulis The tumour tissue was greyish white and pulpy involving both soft tissue and bone All macroscopic pathological bone tissue was removed with a chisel Healing occurred without conplication in a few days Histologic diagnosis adenocarcinoma metastaticum (Fig 2)

The past medical history revealed that five and a half years previously (June 1951) the patient had a Block Mikulicz resection performed for adenocarcinoma in the sigmoid flexure of the colon The patient felt well until the spring of 1954 when increasing symptoms of bronchitis occurred X rays showed a large tumour in the upper lobe of the left lung and in August a left pneumectomy was performed The lung was found to be the site of an extremely pulpy, greyish irregular poorly defined mass measuring 8 by 8 by 8 cm and in the lymph nodes of the hilus numerous whitish infiltrates some measuring up to one cm were seen Microscopy showed an adenocarcinoma probably derived from the colon carcinoma The patient felt well again until December 1956

Following extirpation of the gingival metastasis the condition of the patient was rapidly declining and he died four months later Autopsy revealed a white tumour in the mandible to the right of the midline It was 25 by 20 by 16 mm extending into the gingiva Further findings included osseus metastases to the centre of the eleventh thoracic vertebra and the third lumbar vertebra the ninth rib and the entire right half of the pelvis Soft tissue metastases to lymph nodes were seen throughout the abdomen in the chest on both sides of the neck in the thyroid gland both of the adrenal glands liver pancreas right lung and right pleura Symptomes of recurrence were not demonstrable in the colon

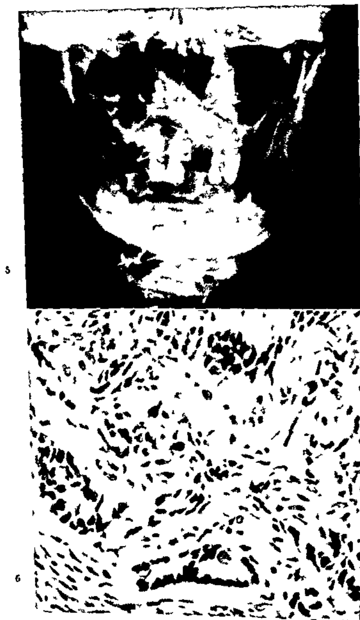
Case 2

On the 5th April 1957 a man aged 77 was referred to the surgical department by his physician because of pains and swelling on the left side of the lower jaw Examination showed a fistular formation in the area of — 4 at the site of a previous operation (in 1955 surgical treatment for a cyst) Radiography showed a sclerosing process in the region of — 5 to — 8 which was interpreted as osteitis or metastasis (Fig 3) During the following weeks a walnut sized fluctuating swelling developed On the 11th April incision was made with evacuation of blood stained pus Culture showed a few colonies of non haemolytic streptococci The swelling persisted and on the 4th June incision was repeated with draining of more pus With a sharp spoon a section of tissue was removed for microscopic examination showing adenocarcinoma (Fig 4)

The past history revealed that the patient had been prostatectomized in January 1955 Diagnosis adenocarcinoma prostatae In October 1955 he was re hospitalized because of pains in the back Radiographic examination showed extensive osteosclerotic metastases Treated with stilbestrol with good response

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Figs 5 and 6

Fig 5 Case No 3 Radiogram of the left side of the lower jaw of a 63 year-old man Resorption of bone is seen in the angle of the mandible progressing towards the condyle and the coronoid process

Fig 6 Case No 3 The histological examination *of the* metastasis shows solid and adenomatous carcinoma Haemalum – eosin × 320



3

4

Figs 3 and 4

Fig 3 Case No 2 Radiogram of the left side of the lower jaw of a 77 year old man. The figure shows a radiopaque process in the region of the lower left second premolar and backwards to the region of the third molar

Fig 4 Case No 2 The biopsy shows adenocarcinoma Haemalum eosin × 320



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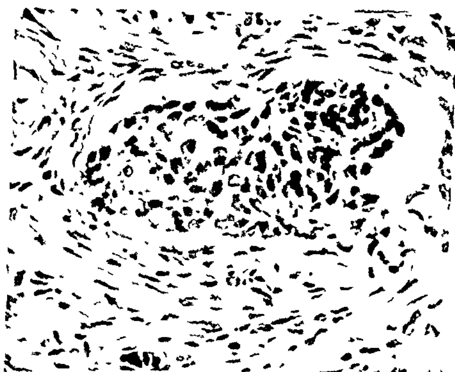
Figs 9 and 10

Fig 9 Case No 5 The radiogram of a 34 year old man shows a destruction of the bone in the region of the bicuspids and the canine of the right side of the mandible

Fig 10 Case No 5 Microscopic examination of the metastasis shows anaplastic carcinoma Haemalum eosin × 370



7



8

Figs 7 and 8

Fig 7 Case No 4 The left side of the lower jaw of a 45 year old woman shows a radiolucent area corresponding to the region of the bicuspids and the first molar

Fig 8 Case No 4 The histological picture shows a solid carcinoma Haemalum eosin × 320



9



10

Figs 9 and 10

Fig 9 Case No 5 The radiogram of a 54 year old man shows a destruction of the bone in the region of the bicuspids and the canine of the right side of the mandible

Fig 10 Case No 5 Microscopic examination of the metastasis shows anaplastic carcinoma Haemalum – eosin × 320

Case 3

The patient was a woman aged 63 who came to the surgical department on the 18th May 1960, referred by her dentist She complained of pains round the left zygomatic arch and anaesthesia and paraesthesia of the lower jaw A small bone intumescence in the region of — 3 4 was removed Temporary improvement followed but as the symptoms recurred and the radiographs gave rise to an suspicion of metastasis to the ramus of the mandible (Fig 5) a biopsy was taken from the area of — 8 The microscopic examination showed carcinoma solidum in partim adenomatosum (Fig 6)

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development of the pathological process

Case 4

A 45 year old woman visited a dentist in the beginning of February 1953 complaining of pains in the region of — 4 The radiograph showed rarefaction apically and — 4 was removed (Fig 7) As the pains continued as before she was referred to a surgical department where a biopsy was taken on the 11th February The histologic diagnosis was carcinoma solidum metastaticum (Fig 8)

The previous history revealed that the patient had been subjected to a right simple mastectomy in August 1952 followed by X ray therapy, administered for the last time on the 16th September 1952 Microscopic diagnosis Carcinoma solidum The patient did well until the symptoms of the jaw metastasis occurred

X ray treatment was given in February March 1953 to the left side of the mandible followed by complete relief of the pains Already at the end of March new metastases appeared subcutaneously on the abdomen and during the following months the patients rapidly deteriorated and died on the 14th June 1953 At autopsy extensive metastases were found in the vertebral column together with metastases at many sites in the subcutaneous tissue Further the liver had greatly enlarged and converted into confluent metastatic tumours

Case 5

The patient was a 54 year old man who on the 2nd March 1958 complained of hypaesthesia of the trigeminal region on the right side most pronounced in the ramus of the jaw After some time a swelling developed in the area of 5 4 — accompanied by paraesthesia and very severe neuralgic pains Radiograms aroused suspicion of metastasis (Fig 9) and histological examination showed anaplastic carcinoma (Fig 10)

Previously the patient had been in general good health Since the end of 1957 increasing cough with dyspnoea developed together with weight loss The sputum contained undifferentiated tumour cells On the 24th February 1958 exploratory thoractomy was performed A tumour the size of a fist was found in the left upper lobe with conglomerates of lymph nodes in the hilus and along the trachea The postoperative course was uneventful except for the abovementioned hypaesthesia on the right side of the mandible

As the pains in the area of 5 4 — caused the patient great distress the mental nerve was severed on the 16th May 1958 The patient rapidly deteriorated and died at home three weeks later

Analysis of Literature and Own Cases

The results obtained from the entire material, *i e* the cases from the literature in addition to our own cases, are presented below

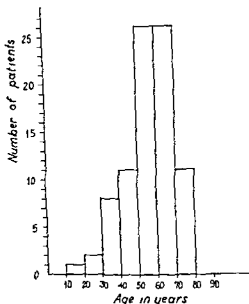


Fig 11
Age incidence of carcinoma metastasis to the jaws 97 patients. First age group is from birth to 9 years, second group from 10 years to 19 years, etc.

Of the total number of 97 cases here considered information of sex was absent in 12, the remaining cases being distributed on 52 women and 33 men.

The age distribution in the 85 cases of which information was obtainable appears in Fig. 11, where the patients are grouped in 10 year periods. The average for all the patients was found to be 55.6 years, for women alone 53.1 years, for men 58.5 years. The median age was 58 years, the youngest patient was 16 years and the oldest 79. For women alone the median age was 57 years, for men 60 years.

Twenty six of the patients consulted first a dentist for the jaw affection, seven a physician, while it remains obscure to whom the remaining 64 patients applied.

Except for swelling and pain information was scarce, of symptoms caused by the jaw metastasis. The distribution of recognized symptoms is shown in Table 1.

TABLE 1
Symptoms of Metastatic Carcinoma of the Jaws in 97 Patients

	Present	Absent	No informations
Swelling	65	5	27
Pain	48	11	38
Anaesthesia	18	1	78
Loosening of teeth	14	20	63
Paraesthesia	7	6	84
Hyperaesthesia	0	8	89
Jaw fracture	6	22	69

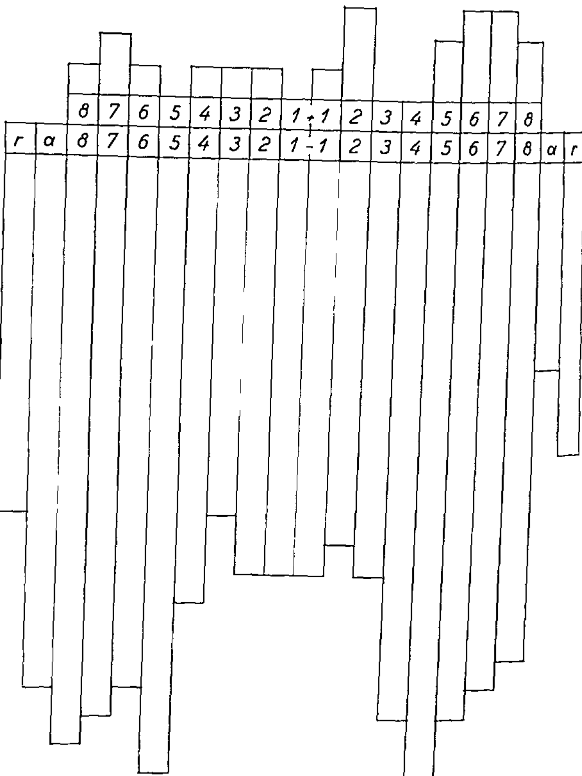


Fig 12

Localization of the metastasis from carcinoma to jaw in 70 cases A square has been marked for each region affected Most metastases cover more than one square The figures indicate the region according to Haderup's nomenclature + designates the upper jaw, and — denotes the lower jaw If the sign is placed to the right of the figure (7+, for example) the right side is indicated and vice versa In this figure "a" designates angulus mandibulae and "r", ramus mandibulae

Among the 65 patients with swellings six presented prosthetic troubles and of the 20 patients who stated to have no loosening of teeth 19 were found to be edentulous in the region affected

The interval between the occurrence of the first symptom of the metastasis and the establishment of the diagnosis varied much from patient to patient In most of the cases symptoms had been present for a few weeks only, while symptoms in some cases had persisted for several months, in a few, from two or three years

Concerning the course of the disease the data showed that in 33 of the cases the jaw metastasis was accompanied by symptoms and diagnosed before recognition of the primary tumour, while the metastasis in 51 of the cases occurred subsequent to the recognition of the primary tumour Information in this respect was lacking in 13 of the cases

In 10 cases the initial manifestation of a jaw involvement had occurred within less than one year after treatment of the primary lesion, in 11 cases one or two years had passed, in 15 cases two to five five years and in six cases five years or more, while information as regards this time interval was absent in 55 cases All groups were represented by tumours arising in widely different organs, nad no type of tumour seemed to have a particularly long or short time interval In the great majority of cases death occurred shortly after the development of the metastasis

The course of the condition after the recognition of the jaw metastasis was recorded in 70 of the patients Of these 62 had died, death occurring within one year in 49 cases within two years in six, and within four years in two cases Of the eight patients who were alive at the time of publication of the case, six had been controlled for one year and one for 15 months, while the last (thyroid carcinoma) presented no evidence of recurrence after seven years

The various sites of origin are listed in Table 2

TABLE 2

Location of Primary Tumour in 97 Cases of Metastatic Carcinoma of the Jaws

Mammae	30
Pulmones	17
Renes	15
Glandula thyreoidea	6
Prostata	6
Colon (including rectum)	6
Ventriculus	5
Melanocarcinoma	5
Testes (2 seminomas 1 embryonal carcinoma)	3
Vesica urinaria	1
Hepar	1
Cervix uteri	1
Ovaria	1
Total	97

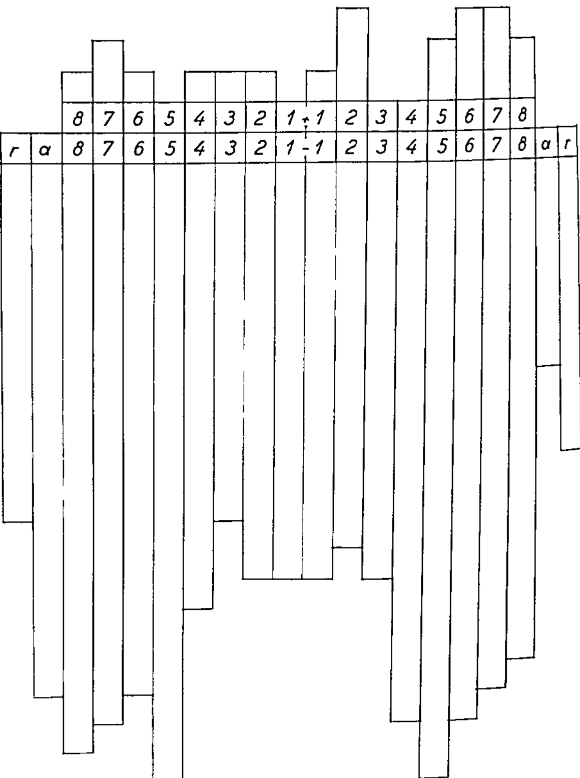


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[illegible]	6
[illegible]	5
[illegible]	5
[illegible]	3
[illegible]	1
[illegible]	1
Cervix uteri	1
Ovaria	1
Total	97

Among the 97 cases 60 presented solitary metastases to the jaws, nine presented multiple metastases, whereas no information is available in 28 cases In 17 patients the upper jaw was involved, in 77 the lower jaw, while two patients presented metastases to both jaws Only in one case such information was not available

In 70 instances the exact localization and extent of the jaw metastases were known, in order to demonstrate a possible predilection the localization of all of the tumours are graphically represented in Fig 12 Each region affected is represented by one square Most tumours cover more than one square As seen in the figure the metastatic carcinomas occurred chiefly in the lower jaw, especially distally of the canines

Objectively, infiltration of the surrounding soft tissues was demonstrated in 37 patients, while the tumour tissue was confined to the bone proper in eight (in 52 cases the sides of affection are unknown)

In 42 patients other bones besides the jaws were affected, while skeletal metastases were localized to the upper or lower jaw in 13 patients In the remaining cases data in this respect are lacking

Metastasis to the lungs was seen in 19 cases, the lesion being absent in 16, and in 62 cases data in this report were not available

No definite difference could be demonstrated as regards survival times in groups of patients presenting involvement of the lungs or of various bones, and patients presenting jaw metastasis exclusively

DISCUSSION

Only few comprehensive analyses are available from the literature on metastatic carcinoma to the jaws, such phenomena being published chiefly in the form of case reports *Richard et al* 1956, have given a review of the literature between 1892 and 1956, and *Castigliano & Rominger* 1954, have reviewed the literature from 1902 to 1953

As shown by our results only 92 cases from the literature were found to fulfil the criteria set up by us In a previous work (*Castigliano & Rominger* 1954) a higher number has been reported In their review of the literature they found a total of 176 cases of metastatic involvement of the jaws

This great difference in number of cases selected must be ascribed partly to the inclusion by *Castigliano & Rominger* of metastatic sarcomas Further they included cases of lip carcinoma and carcinoma of the thyreoid gland which often lacked adequate data as judged by our criteria, or which proved to be cases of direct invasion of a primary tumour to the jaw In many other cases there was no histologically verified diagnosis of the metastasis or reliable information of the diagnosis or location of the primary tumour Finally, *Castigliano & Rominger* included cases of metastases to the soft gingival tissues

As seen in Fig 11 the age distribution of the material here discussed is in fair accordance with previous findings in cases of metastases to

the jaw bones (*Richard et al* 1956, and *Cash et al* 1961) and of osseous metastases in general (*Geschickter & Copeland* 1949) Thus, with regard to time of occurrence metastatic lesions in the jaw do not seem to differ from other skeletal metastases

In a series of 20 persons *Cash et al* found an average age of 56 7 and a mean age of 60 These figures are in perfect agreement with the findings by the present authors

There is a considerable difference between the number of women and men in our cases, and this difference, which is significant, $p < 0\ 01$, (*Fisher* 1954), is undoubtedly due to the fact that metastatic carcinoma from the breast accounts for about 30 per cent of all metastases included The series reported by *Cash et al* included nine women and 11 men, only two of these 20 metastases were derived from mammal carcinoma

The most frequent symptoms of metastatic carcinomas of the jaws are swelling and pain—as in other bone metastases The diagnostic value of these symptoms is impaired by the frequency with which they occur in dental diseases in general More characteristic features of mandibular metastasis are the anesthesia and paraesthesia involved, these two symptoms being present only rarely in other pathological conditions Their importance are diminished, however, by the fact that they were stated to occur only in about 25 per cent of the cases included The importance of sensibility disturbances has been demonstrated, in particular, by *Roger & Paillas* 1937 As opposed to anaesthesia and paraesthesia no case of hyperaesthesia was reported

Another, not uncommon, symptom is the loosening of one or more teeth in the same area This symptoms in particular is significant in patients with intact teeth and absence of generalized periodontitis

Spontaneous jaw fractures are rare, even in advanced cases, if present they will probably lead to a prompt and correct diagnosis

Although the incidence of metastases to the jaw in the frontal region of the mandible apparently is inferior to the incidence distally of 3–3, this cannot be regarded as evidence of a predilection There is, however, a much higher frequency of development of tumours in the mandible than in the maxilla, and this distribution is in good agreement with previous works

The course of the disease must be described as relatively rapid Thus most of the patients will only have symptoms persisting for one to two months before the metastasis is discovered

Summarily it can be said that recognition of metastasis to the jaw bones is of special interest, because in a great number of cases (in the present series, 33 out of 97) the involvement of the jaw presents the first symptom of tumour at all

It must be concluded that metastasis to the jaw bones must be suspected in particular, if patients with swelling and pains in these areas

show signs of anaesthesia as well as paraesthesia Loosening of teeth and spontaneous jaw fracture are also significant symptoms

Radiography does not give characteristic findings, and may be quite negative at the initial stage of a metastasis to the jaw Therefore biopsy is essential for an establishment of the diagnosis metastatic carcinoma of the jaws

SUMMARY

The literature from 1884 to 1961 dealing with metastatic carcinoma of the jaws has been reviewed Ninety seven cases have been selected in accordance with the criteria established by the authors Ninety two have been collected from the literature, and five of our own cases have been added

The frequency of metastatic carcinoma of the jaws was found to be higher among women than among men, possibly because metastasis from carcinoma of the breast constituted nearly one third of the total number of cases The average age for all the patients was 55 6 years, being somewhat higher for men and somewhat lower for women

The most common symptoms were found to be swelling and pain but the most significant symptom was paraesthesia of the lower jaw In about one third of the cases the metastasis gave symptoms and was recognized before the primary tumour The prognosis was found to be very poor, 70 per cent of the patients died within the first year after detection of the metastasis

The majority of the metastatic lesions were derived from carcinoma of the breast, lung, and kidney The most common location was found to be the lower jaw, especially distally of the canines

Radiography gives rather uncharacteristic findings, hence biopsy is essential for the establishment of a correct diagnosis

REFERENCES

Cash C D Royer R Q & Dahlin D C Metastatic tumors of the jaws Oral Surg *14* 897 905 1961

Castigliano S G & Rominger C J Metastatic malignancy of the jaws Amer J Surg *87* 496 507 1954

Fischer R A Statistical methods for research workers 13th edition 356 pp Oliver & Boyd Edinburgh 1958

Geschickter C I & Copeland M M Tumors of the bone 3rd edition 810 pp Lancaster Press Lancaster 1949

Haderup V Vorschlag zu einer internationalen Bezeichnung der Zähne Korresp Bl Zahnärz *16* 314 1887

Richard A Cernéa P Hennion P Commissionat Y & Marie M P Les épithéliomas métastatiques de la mandibule Rev Stomatol *57* 652 673 1956

Roger H & Paillas J E Le signe du mentonnier (paresthésie et anesthésie unilaterale) révélateur d un processus néoplasique métastatique Rev neurol *68* 751 752, 1937

FROM CANCERREGISTERET AND DEPARTMENT OF PATHOLOGY, FINSEN INSTITUTE COPENHAGEN DIRECTOR JOHANNES CLEMMESEN M.D

STUDIES ON THE NEWT TEST FOR CARCINOGENICITY

3 *Non Carcinogenic and Weak Carcinogenic Hydrocarbons*

By

E ARFFMANN

Received 10 x 62

Studies on the specificity of a test for carcinogenicity should be performed by comparing the action of carcinogens to related non-carcinogenic compounds Among polycyclic aromatic hydrocarbons the best known non-carcinogens are phenanthrene and anthracene These two chemical compounds are of a rather simple constitution, and it should seem particularly interesting to examine non-carcinogenic hydro carbons of a more complex structure closer to potent carcinogens The experimental experience on such compounds, however, is quite small as compared to that on strong carcinogens like benzo[*a*]pyrene, dibenz-[*a h*]anthracene and 3-methylcholanthrene, so that it is open to doubt whether they are really non-carcinogenic, *i e* inactive under all experimental conditions This uncertainty is increased by the general consideration that it is extremely difficult, or impossible, to draw a sharp line between carcinogens and non-carcinogens, the difference probably being more quantitative than qualitative and also dependent on the given conditions These circumstances make it more adequate to work along a quantitative line in the present investigations on the specificity of the newt test and so examine the effect of simple non-carcinogens and weak carcinogens as compared to that of related strong carcinogenic hydrocarbons

The hydrocarbons chosen for the present experiments are shown in Table 1 It includes 3',6 dimethyl benz[*a*]anthracene as a representative of the complex supposedly non carcinogenic hydrocarbons

REPORTED RESULTS IN MAMMALS

Phenanthrene Since the negative results of *Kennaway* (1924), *Kennaway & Hieger* (1930) and *Maisin et al* (1926 1927) [illegible]

[illegible]e author's thanks are due to Professor *F Boyland*, London and Professor *H Druckrey* Freiburg for valuable suggestions and help

show signs of anaesthesia as well as paraesthesia Loosening of teeth and spontaneous jaw fracture are also significant symptoms

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The most common symptoms were found to be swelling and pain, but the most significant symptom was paraesthesia of the lower jaw In about one third of the cases the metastasis gave symptoms and was recognized before the primary tumour The prognosis was found to be very poor, 70 per cent of the patients died within the first year after detection of the metastasis

The majority of the metastatic lesions were derived from carcinoma of the breast, lung, and kidney The most common location was found to be the lower jaw, especially distally of the canines

Radiography gives rather uncharacteristic findings, hence biopsy is essential for the establishment of a correct diagnosis

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Castigliano S G & Rominger C J Metastatic malignancy of the jaws Amer J Surg *87* 496–507 1954

Fischer R A Statistical methods for research workers 13th edition 356 pp Oliver & Boyd Edinburgh 1958

Geschickter C F & Copeland M M Tumors of the bone 3rd edition 810 pp Lancaster Press Lancaster 1949

Haderup V Vorschlag zu einer internationalen Bezeichnung der Zahne Korresp Bl Zahnarz *16* 314 1887

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Roger H & Paillas J F Le signe du mentonnier (paresthésie et anesthésie unilaterale) révelateur d un processus néoplasique métastatique Rev neurol *68* 751 752 1937

purified chrysene yielded no sarcomas in a total of 100 mice, observed for 11 to 12 months The degree of carcinogenicity is stated as being very slight This statement is confirmed by *Barry et al* (1935), who induced 1 epithelioma and 3 papillomas in 150 mice painted twice weekly with a 0 3 per cent solution in benzene *Barry & Cook (1934)* injected an impure sample of chrysene subcutaneously and saw 4 mesenchymal tumours in 10 rats several being definitely malignant The result was negative in mice In an aqueous colloidal solution purified chrysene had no carcinogenic effect on the subcutis of rats (*Boyland & Burrows* 1935) *Pollia* (1939, 1941) observed his painted mice for only 133 days and made injections on only 5 rats No tumours were seen

The first experiments with pure or synthetic chrysene were largely negative, both by painting of mice (*Schürch & Winterstein* 1935) and by subcutaneous injection in mice (*Shear & Leiter* 1941) The result of *Bachmann et al's* experiment (1937), however may indicate that a long period of observation is necessary, their first and only epithelial tu ... inted mice lived for more than 44 ... 10 rats of solutions of chrysene ... Cook et al 1936) In 1951 *Riegel* ... carcinoma among 15 surviving mice painted for 31 weeks with 0 2 per cent chrysene In the same year *Steiner & Falk* (1951) ascertained that chrysene is a weak carcinogen They obtained 4 sarcomas in 24 surviving C57 black mice injected subcutaneously with 5 mg chrysene in tr ...

... a series of injecti ... genic properties of anthracene (*Fibiger* 1923 *B* ... *Bergol* ts & *Hygina* 1951) Only in a few instan ...

injec ... als were Five ... ly doses Four ... njection runge ... Wuche- simil ... In a ... intraperitoneal injections were given and one out of 5 surviving rats got a metastasizing spindle-cell sarcoma 2 years later So the non carcinogenicity of anthracene seems to be qualified

Benz[a]anthracene According to painting experiments the carcinogenicity of benz[a]anthracene is very slight One epithelioma was provoked among 20 ... *Barr* ...

... anthracene have been used for subcutaneous injections in rats (*Boyland & Burrows* 1935) and in 1 rabbit (*Hoch Ligeti* 1941) Tumours were not noted *Klein* (1952) provoked a carc ... muscular inj ... and ... benza ... in tr ... depen ... was t ... uction of 43 sarcomas among 180 mice (effective total) Average induc

tion time varied from 236 days to 438 days In conclusion benzanthracene was characterized as moderately carcinogenic under the conditions of the experiments

White & Fschenbrenner (1945) found hepatomas in 2 out of 6 rats fed benz[*a*] anthracene for 9 to 14 months The occurrence of tumours seems not to be casual *Shear & Leiter* (1941) after one year observed a raised number of tumour nodules in the lungs of two strain A mice injected with benzanthracene but *Andervont & Shimkin* (1940) did not find this substance able to induce pulmonary tumours after 14 to 20 weeks

3 6 Dimethyl benz[a]*anthracene* is generally accepted as a non carcinogen (*Cook et al* 1936 *Badger* 1948 *Chalvet & Mason* 1961) This view seems to be founded on one experiment described by *Bar* ... painted on the back with the hy... 1 year the last one dying on the ... related 2 6 dimethyl and 3 monomethyl derivatives were inactive too while the application of 6 methyl benz[*a*]anthracene led to the appearance of 2 epitheliomas and 1 papilloma in 10 mice The number of animals in these tests is inadequate for final conclusions but the results on the two monomethyl derivatives have been confirmed in recent, more extensive experiments (*Haam* reported by *Newman & Otsuka* 1958 *Dunning & Curtis* 1960)

TECHNIQUE

The applied technique has been described in earlier reports (*Arffmann & Christensen* 1961 *Arffmann* 1962) and only slight modifications have been introduced during the present investigations A small notch made with scissors has replaced the suture as a mark of the level of needle puncture in the tail This procedure is easier to perform and gives a safer marking than the suture which often fell off

In a period when available newts were few the author tried to use subcutaneous injections in the back of salamanders whose tails had previously been amputated after testing by the usual method This new technique has formerly been applied by *Koch et al* (1939) and by *Neukomm* (1944) both obtaining epidermal hyperplasia and infiltrative downgrowth on the injection of carcinogenic solutions In the present experiments injections were made to the right of the dorsal midline just in front of the hind leg The needle was introduced obliquely and the deposit amounted 0 05 ml forming a small prominence of the overlying skin The time of observation followed the usual scheme the animals finally being killed and fixed in toto After fixation the area of injection was isolated by transverse cuts through the whole body and preparated as earlier described

Another way of utilizing once injected newts was tried in a few experiments Salamanders with partially or wholly regenerated tails were chosen and the injection made in the proximal part of the tail just distal to the anus This region had not been amputated at the first testing and so represented the original and not the secondary tail

The various test solutions were prepared in the usual way (*Arffmann & Christensen* 1961) The hydrocarbons were carefully pulverized and so the period of heating for the less soluble compounds could be reduced to 1 15 minutes Refined peanut oil and soyabean oil served as solvents new samples being delivered every month from Dansk Sojakagefabrik The peroxide value was controlled and the oils were kept in icebox under nitrogen atmosphere

Phenanthrene puriss (L Light & Co England) and 1 2 4 trimethylphenanthrene (supplied by the courtesy of Prof H Dannenberg Munich) were easily soluble in the oils Anthracene (The British Drug Houses Ltd) triphenylene (L Light & Co) benz[*a*]anthracene (L Light & Co) and 3 6 dimethyl benz[*a*]anthracene (supplied by the courtesy of Prof A Haddow London) required a short heating for the establishment of clear solutions Most difficult to solve was chrysene (L Light & Co) and like anthracene the 0 5 per cent solution needed a second heating before injection because of secondary precipitation The pure oils used for controls were heated if the test solutions were

The experimental animals (Triton cristatus) were as far as possible divided equally according to sex

As to the tissue blocks it was found by comparison that every 15th of the serial sections sufficed for the histologic demonstration of even minute lesions This has effected a considerable reduc... number of microscopic slides

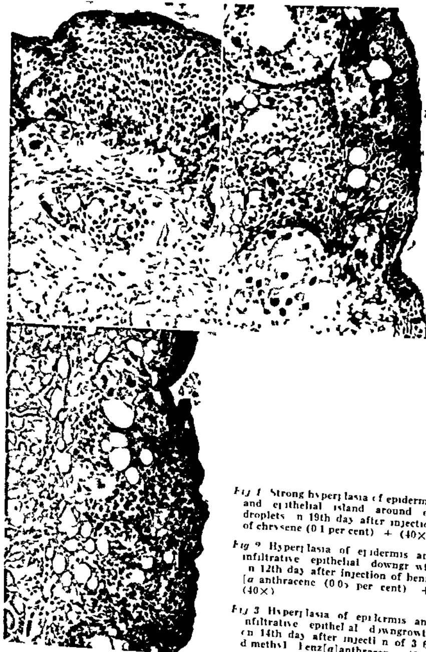


Fig 1 Strong hyperplasia of epidermis and epithelial island around oil droplets on 19th day after injection of chrysene (0.1 per cent) + (40×)

Fig 2 Hyperplasia of epidermis and infiltrative epithelial downgrowth on 12th day after injection of benz[*a*]anthracene (0.05 per cent) + (40×)

Fig 3 Hyperplasia of epidermis and infiltrative epithelial downgrowth on 14th day after injection of 3,6-dimethyl benz[*a*]anthracene (0.05 per cent) + (40×)

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painted on the back with the hydrocarbon
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Trimethylphenanthrene

		26						38						46					
… 0.1% in peanut oil		Soyabean oil		Phenanthrene 0.1% in soyabean oil		1,2,4-Trimethylphenanthrene 0.1% in soyabean oil		Peanut oil		Phenanthrene 0.5% in peanut oil		1,2,4-Trimethylphenanthrene 0.5% in peanut oil		Peanut oil		1,2,4-Trimethylphenanthrene 0.5% in peanut oil		Chrysene 0.5% in peanut oil	
		Tail						Tail						Part of Tail Proximal to Secondary Tail					
8		8		8		8		6		6		6		3		5		5	
♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
											–			–					
–	–	–	–	–	–	–	–											+	
	–	–	–	–	–	–	–	–	–	–	–	– –			±	– –			+
–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–		– –					–	±	
0		0		0		0		0		1 (included)		0		1 (included)		0		1 (included)	

response in any case An increase in strength of the injected solution from 0.1 to 0.5 per cent did not influence this result A single (+) reaction to triphenylene in 0.1 per cent solution was not different from what may be seen after the injection of peanut oil (cfr exper No 30) or soyabean oil In each of the first two experiments (No 27 and 28) chrysene had a frankly positive effect in one animal, while later experiments more convincingly showed its moderate or sometimes even rather strong activity (Fig 1) Anthracene (Table 4) was negative with one exception at a concentration of 0.5 per cent Benz[*a*]anthracene like chrysene induced a weak or moderate positive response (Fig 2), while the injection of 3',6-dimethyl benz[*a*]anthracene was followed by a strong positive reaction slightly inferior to that of dibenz[*a,h*]anthracene (Fig 3) This surprising result was first seen in two experiments (Table 5) where some controls were positive, but a later experiment (No 32) confirmed the finding with half the concentration of the hydrocarbon

One experiment (No 33) was intended to show whether the inhibitive

TABLE

Experiments with Phenanthrene and

Experiment No	23			24			25	
Substance	Peanut oil	Phenanthrene 0.1% in peanut oil	1,2,4 Trimethylphenanthrene 0.1% in peanut oil	Soyabean oil	Phenanthrene 0.1% in soyabean oil	1,2,4 Trimethylphenanthrene 0.1% in soyabean oil	Peanut oil	Phenanthrene 0.1% in peanut oil
Site of Application	Tail			Tail			Tail	
Number of Animals (Triton cristatus)	8	8	8	8	8	8	8	8
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
Epidermal Reaction on								
3–4 days								
9–10 days	— —	— —	— —	— —	— —	— —	— —	— —
12 days	— —	— —	— —	— —	— —	— —	— —	— —
15 days	— —	—	— —	— —	— —	— —	— —	— —
18 days	— —	—	— —	— —	— —	— —	— —	— —
Animals died during experiment	0	0	0	0	0	0	0	0

RESULTS

The results of injection experiments on newts with the above-mentioned non carcinogens and weak carcinogens (Table 1) are given in Tables 2-5 and 7. Each compound has been submitted to several separate tests, and in most experiments the effect of closely related inactive and weakly active substances has *been compared, also to* pure oils and in some instances to strong carcinogenic hydrocarbons.

As in previous experiments the histological examination, performed by the author personally, showed no constant correlation between macroscopic findings and histological picture. In positive cases the skin may be normal or prominent, while strong responses are often accompanied by visible ulcerations. The quantitative estimation of the results is based on the incidence of partly positive and positive reactions, not on the degree of these. This principle tends to reduce subjectivity.

Phenanthrene and 1,2,4-trimethylphenanthrene induced no epithelial

henylene and Chrysene

30		31-32				37			
0.1% in peanut oil	Triphenylene 0.1% in peanut oil	Soybean oil	Chrysene 0.5% in soybean oil	Triphenylene 0.1% in soybean oil	Dibenz(a,h)anthracene 0.05% in peanut oil	Peanut oil	Chrysene 0.5% in peanut oil	Triphenylene 0.5% in peanut oil	Benz(a)pyrene 0.5% in peanut oil
Tail		Tail				Back			
8	8	8	8	8	8	8	8	8	8
♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
	± —	— —	(+)	—	— +	—	—	—	—
+	—	— —	+ —	— —	+ +	— —	+ +	— —	— —
(+) +	—	— —	— +	— —	— +	— — —	+ ±	— — — —	— + — ±
+	— —	— ±	— —	— —	+ +				
0	0	0	0	1	0	3 (2 included)	4 (1 included)	1 (included)	1

direct continuity with the overlying epidermis (Fig 4) The explanation seems to be that the proliferating epithelium follows the route prepared by the needle of injection, a course already described by *Koch et al* (1939) as typical after 1 injection In one case, however, after the injection of benzpyrene a vigorous epithelial hyperplasia and direct downgrowth was seen in the midline of the back (Fig 5)

The other site of application in secondly used salamanders was the tail proximal to the first amputation In most of these cases old cysts surrounded by a capsule of connective tissue were found as remnants of the earlier injection (Fig 6) The residue has obviously not affected the outcome

Table 7 shows an experiment made at a time of the year, when the few remaining newts were in a bad condition of health Generally they were less vivacious, and in many the skin was set with numerous white spots *presumably* due to some infection Microscopically these lesions showed localized thickening and splitting up of the epithelium with

TABLE

Experiments with

Experiment No	27						28-29									
Substance	Peanut oil		Chrysene 0.1% in peanut oil		Triphenylene 0.1% in peanut oil		Soyabean oil		Chrysene 0.1% in soyabean oil		Triphenylene 0.1% in soyabean oil		Benzopyrene 0.1% in soyabean oil		Peanut oil	
Site of Application	Tail						Tail									
Number of Animals (Triton cristatus)	8		8		8		8		8		8		8		8	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal Reaction on																
6–7 day														+		
9–10 day	—	—	—	—	—	—	—				—		(+)		—	—
12 day	—	—	—	—	—	—	—		—	—	—	—	—	+	—	+
14–15 day	—	—	—	—	(+)	—	—	—	—	+	—	—	—	—	—	—
18–19 day		—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
Animals died during experiment	0		0		0		0		0		0		1 (included)		0	

effect of a weak carcinogen (benz[*a*]anthracene) on a strong carcinogenic hydrocarbon (dibenz[*a,h*]anthracene), demonstrated in an injection experiment on mice by *Steiner & Falk* (1951), could be reproduced in the salamander The applied solution contained 1 mg of benzanthracene and 1 mg of dibenzanthracene in 1 ml of peanut oil and was injected into 8 salamanders The incidence of positive reactions was, however, not significantly lower than that obtained with dibenzanthracene alone

Second use of the salamanders by two different ways of application was found possible (Table 6), and the results obtained by these measures have been incorporated together with results obtained by the usual technique The use of subcutaneous injection into the back, however seems to be a less sensitive method In addition, the histological picture in positive cases is slightly different from that found in tails and less pronounced Most often hyperplasia is moderate and epithelial downgrowth is recorded as epithelial islands, often ringshaped and not in

ınd 3 6 Dımethyl ben-[a]anthracene

38		32				33			
I eanut oil	Anil racene 0 3% in pean ut oil	Peanut oil	Benz[a anthracene 0 05% in peanut oil	3 6-Dime th ylbenz[a] anil racene 0 05% in peanut oil	Dil enz a h anthracene 0 05% in peanut oil	Pea nut oil	Benz a]-anil racı ne 0 1% in peanut oil	Benz a an thrac 0 1% + Dil enz[a h] anthrac 0 1% in pean it oil	Dil enz a h]-anthracene 0 1% in I eanut oil
Tail		Tail				Tail			
6	6	8	8	8	8	8	8	8	8
♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
		—							+ ±
		—	— —	— +	— +	— —	— +	— —	+ +
—	+ —	— —	+ —	(+) —	+ +	— —	+ +	+ +	+
—	—	— —	— +	— / + +	— +	— —	— —	+ +	+
—	—	—	—	+	+ +	— ±	— —	+ +	+ +
0	0	1 (included)	0	1 (included)	0	0	0	0	2 (included)

0 1 per cent solution 3 methylcholanthrene has a stronger effect, and this is slightly augmented by increasing the concentration to 0 5 per cent The few similar experiments with weak carcinogens indicate, too, an increase in response parallel with the strength of the solution

DISCUSSION

Epidermal proliferation in the newt is not a reaction specific to carcinogens which was to be expected No doubt, *Lucke & Schlumberger* (1949) are right in regarding the lesion as a non-neoplastic hyperplasia *resulting from prolonged irritation*" We experienced this finding in the earlier experiments a vigorous downgrowth of hyperplastic and ill-defined epithelium around the inserted suture This circumstance, however is of minor importance as regards the applicability of the reaction as a quick test, if the method can distinguish between carcinogens and non-carcinogens and perhaps even yield some quantitative information

In the present investigation non carcinogens and weak carcinogens

TABLE

Experiments with Anthracene Ben-[a]anthracene

Experiment No	39											
Substance	Soyabean oil				Anthracene 0 1% in soyabean oil				Benz a anthracene 0 1% in soyabean oil			
Site of Application	Tail		Back		Tail		Back		Tail		Back	
Number of Animals (Triton cristatus)	4		4		4		4		4		4	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal reaction on												
4 – 5 day			—									±
8 – 10 day	—				—		—		—			
12 day		±		—	—			-	—		—	
14 – 15 day	—		—		—		—		+		—	
18 day	±			—	-			—		+		—
Animals died during experiment	0		0		0		0		0		1 (includ d	

degenerative changes of the cells, especially in the middle where necrosis led to a small defect of the surface Among and in the degenerated cells were many small, round or oval, pale bodies, supposed to be protozoes Investigations on the living material for an exact diagnosis of the parasites have not been performed The results of the experiment were not clear, due to the high mortality and possibly to the infection Triphenylene had a rather strong positive effect at a concentration of 0 5 per cent, but this calls for reassessment

The last Table (8) shows, for comparison with the other reported results, the influence of varying the concentration of the potent carcinogens Experiment No 20 is partly impaired by bad survival of the animals, disturbing the original plan to examine all tails on the *14th day* Yet it clearly shows that dibenz[*a,h*]anthracene has the same strong effect at 1, 2 and 5 mg per ml of oil This is hardly weakened by reducing the concentration to 0 05 per cent (see Table 4) Benzo[*a*]-pyrene was inactive in a 0 05 per cent solution, while the reaction to the 0 2 and 0 5 per cent solutions was a little stronger than that to a

were tested In most experiments a concentration of 0 1 per cent in peanut oil or soyabean oil was chosen and so the results are comparable to those earlier obtained using 3 potent carcinogenic hydrocarbons (*Arffmann & Christensen* 1961) Working with higher concentrations is more difficult because of the weak solubility of some of the hydrocarbons

The biologic processes observed in the newt test exhibit features peculiar to this species of animals and so cannot a priori be compared to a single sort of tests on warmblooded animals for instance skin painting experiments A comparison with results obtained by any way of application in mammals therefore seems adequate

TABLF 5

The First Experiments with 3 6 Dimethyl benz[a]anthracene

Experiment No	18						19					
Substance	Peanut oil*		3 6-Dimethylbenz[a]anthracene 0 1 % in peanut oil		Dibenz[a h]anthracene 0 1 % in peanut oil		Peanut oil*		3 6-Dimethylbenz[a]anthracene 0 1 % in peanut oil		Dibenz[a h]anthracene 0 1 % in peanut oil	
Site of Application	Tail						Tail					
Number of Animals (Triton cristatus)	10		10		10		10		10		10	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal Reaction n												
3 5 day						—		— —			+	
6 7 day				— ±	—							
10 day			—	+	+	+		—	(+)	+	+	†
14 15 day		+	— +		+	+		—	—	+	+	+
20 day	+	—	(+)	+ +§	—	+		(+) —	+	±	(+)	+
38 42 day		—			—	(+)			(+)			
Animals died during experiment	1 (included)		4 (3 included)		1 (included)		5 (2 included)		3		4 (1 included)	

* The oil was heated at 100° C for 15 minutes like the test solutions
§ Dead on the 23rd day
† Sections technically bad

Fig 4



Fig 5





Fig 6

Fig 4 Moderate epidermal hyperplasia and epithelial islands around oil droplets on 12th day after subcutaneous injection of chrysene (0 5 per cent) in the back + (40×)

Fig 5 Strong epidermal hyperplasia and direct epithelial downgrowth in the midline of the back on 15th day after subcutaneous injection of benzo[*a*]pyrene (0 5 per cent) + (40×)

Fig 6 Second use of tail proximal to secondary tail Epithelial hyperplasia and downgrowth on 15th day after injection of dibenz[*a h*]anthracene (0 1 per cent) + Encapsulated cyst from former experiment is seen to the right (25×)

explanations are possible After all, the weak carcinogenicity of trimethylphenanthrene may be questionable, the evidence being only one experiment on 10 mice Another possibility is connected with the great solubility of the hydrocarbon, which may lead to a faster resorption from the subcutis of the newt The same applies to benzo[*a*]pyrene, but this compound is a strong carcinogen and so still induces a definite positive response, even if it is weaker than expected according to its generally accepted position of strength (*Barry et al* 1935, *Iversen* 1949)

TABLE 7

Experiment on Newts with Infected Skin

Experiment No	43							
Substance	Peanut oil		Anthracene 0 5% in peanut oil		Triphenylene 0 5% in peanut oil		Chrysene 0 5% in peanut oil	
Site of Application	Tail							
Number of Animals (Triton cristatus)	6		6		6		6	
Sex	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal Reaction on								
3 4 day		—	—					
6 7 day								+
9 day	+	—						
11 12 day					+ —			
15 day	—		—	+	(+) +		+ +	
18 day					(+) (+)			
Animals died during experiment	4 (3 included)		3		0		4 (1 included)	

3 6 dimethyl benz[*a*]anthracene was found strongly active in the newt test which consequently may not be able to separate clearly non-carcinogens of so complex a structure from closely related carcinogens On the other hand the inactivity of 3,6 dimethyl benz[*a*]anthracene has been demonstrated in only one experiment on 10 mice and so is not definitely ascertained

TABLE 6

Preliminary Experiments on Second Use of the Salamanders

Experiment No	36				41			
Substance	Soyabean oil		Dibenz[a,h]anthracene 0 1% in soyabean oil		Soyabean oil		Dibenz[a,h]anthracene 0 1% in soyabean oil	
Site of Application	Back				Part of Tail Proximal to Secondary Tail			
Number of Animals (Triton cristatus)	8		8		6		6	
Sex	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal Reaction on								
4 day				—				
9 day	—			—				
11–12 day	—	—	—	±	—	—	—	+
15 day		—	+ ±		—	—	—	+
18 day	— —			+		—		+ +
Animals died during experiment	2		3 (2 included)		0		0	

The experimental results here reported on phenanthrene, anthracene, triphenylene at 0 1 per cent, benz[a]anthracene and chrysene are in good accordance with the conclusions to be drawn from the reviewed experiments using warm-blooded animals This means that the newt test is able to distinguish the non-carcinogenic phenanthrene, anthracene and triphenylene from the closely related weak carcinogens benz[a]anthracene and chrysene The sharp distinction is a little blurred by one or two positive reactions to anthracene at 0 5 per cent and by the possible activity of triphenylene at the same concentration These findings may express that the specificity of the newt test is decreasing at higher strengths of the test solutions, or that anthracene and triphenylene are not absolutely non-carcinogenic The latter interpretation is supported by the results of *Druckrey & Schmähl* (1955) on anthracene, and finds no absolute contradiction in the admittedly negative, but also insufficient testing of triphenylene in mammals

Another discrepancy is presented by 1,2,4-trimethylphenanthrene which had no effect on the epidermis of the newt Here, also, different

...nt Carcinogens

		29										31							
Soya bean oil		Benzo a pyrene in soyabean oil								Pea nut oil		3-Methylcholanthrene in peanut oil							
		0 05%		0 1%		0 2%		0 5%				0 05%		0 1%		0 2%		0 5%	
		Tail										Tail							
16		8		8		8		8		16		8		8		8		8	
♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
					+										—				
	—		—	—		(+)	§		—	— —	— —	+	+	+	+		— +	+ +	+
	— —			—	+	—	(+)	+		— —	—	+			+	+		+	
	—			—			±	+	—	— —	— —	+	+	—		+	+		— +
		—	— —	— —		—	§	+		— —	— —	—	+	(+)	+	-	+	+	+
0		0		1 (included)		0		2 (1 included)		0		1		2 (included)		1 (included)		1 (included)	

some extent follows the carcinogenicity of the compound and also the concentration of the test solution

Some supplementary experiments on the specificity of the newt test will be published later

SUMMARY

Some non carcinogenic and weak carcinogenic hydrocarbons have been tested on Triton cristatus Phenanthrene and, with a few exceptions, anthracene and triphenylene gave no epithelial response, while chrysene and benz[a]anthracene induced a positive reaction, estimated as weak or moderate according to the incidence In disagreement with

T

Experiments with Varying Concentr

Experiment No	20								
Substance	Peanut oil	Benzo a pyrene in peanut oil			3 Methylcholanthrene in peanut oil			Dibenz a h a cene* in pe	
		0 1%	0 2%	0 5%	0 1%	0 2%	0 5%	0 1%	0
Site of Application	Tail								
Number of Animals (Triton cristatus)	5	5	5	5	5	5	5	5	
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂
Epidermal reaction on									
3 5 day	—	—		(+)		(+)			
6 7 day				—					
8–10 day			—			+			
12 13 day			—						
14 15 day	— —	+ lost	+ + +	+	{ – — +	— +	{ + + — +	{ + + + + +	
18 19 day									
Animal died during experim	3 (2 included)	3 (included)	2 (included)	4 (2 included)	2	3 (2 included)	1	0	cl

* See also Table 4 No 32 and No 33

§ Minimal signs of the injected oil

As far as anthracene, benz[*a*]anthracene and chrysene are concerned the results are in accordance with those of *Neukomm* (1957) Still, the larger number of animals in the present experiments has made it possible to demonstrate a quantitative difference in response to weak and strong carcinogens like that seen in mammals

The conclusion to be drawn is that the newt test has shown such specificity as to the action of polycyclic hydrocarbons that it may be considered a useful screening test for the carcinogenicity of such compounds Furthermore, the results obtained by potent and weak carcinogens provide suggestive evidence that the degree of response to

Fibiger J État actuel des recherches sur la production expérimentale du cancer les buts de ces recherches et les problèmes qui en prennent leur origine Acta chir scandinav 55 343 362 1923

Graffi A *Vlamynck F* *Hoffmann F* & *Schul I* Untersuchungen über die geschwulstauslosende Wirkung verschiedener chemischer Stoffe in der Kombination mit Crotonol Arch Geschwultsforsch 5 110–126 1953

Hallow A & *Robinson A M* The Influence of Various Polycyclic Hydrocarbons on the Growth Rate of Transplantable Tumours Proc Roy Soc London ser B 122 442 476 1937

Hill W T *Stanger D W* *Pizzo A* *Riegel B* *Shubik P* & *Wartman W B* In in Mice by

Hoch Immunity

Huh nt Brit J

Iversen S A Possible Correlation between Absorption Spectra and Carcinogenicity Thesis Copenhagen 1949

Kennaway E L On the Cancer Producing Factor in Tar Brit M J *1* 564–567 1924

Kennaway E L & *Hieger I* Carcinogenic Substances and Their Fluorescence Spectra Brit M J *1* 1044 1046 1930

Klein M The Action of Methylcholanthrene Croton Oil and 1,2 Benzanthracene in the Induction of Skin Tumors in Strain DBA Mice J Nat Cancer Inst *12* 735 742 1952

Klein M Effect of Croton Oil on Induction of Tumors by 1 2 Benzanthracene Desoxycholic Acid or Low Doses of 20 Methylcholanthrene in Mice J Nat Cancer Inst 13 333 341 1952

Koch C *Schreiber B* & *Schreiber G* Tumeurs produites par le goudron et le benzopyrene chez les amphibiens urodèles Bull Assoc franç p l etude du cancer 28 852–859 1939

Lucke B & *Schlumberger H G* Neoplasia in Cold Blooded Vertebrates Physiol Rev 29 91 126 1949

Maisin J *Rome M* & *Jacqmin L* Methode d obtention de goudron non cancérigène Compt rend soc de biol *94* 767 769 1926

Maisin J *Desmedt P* & *Jacqmin L* Pouvoir cancérigène du Carbazol Compt rend soc de biol *96* 1056–1058 1927

Miescher G Experimentelle Untersuchungen über Krebserzeugung durch Photosensibilisierung Schweiz med Wchnschr 23 1082 1084 1942

Neukomm S Le problème de la cancerisation par le goudron et les substances cancérigenes chez les Tritons Memoires de la Societe Vaudoise des Sciences naturelles Lausanne 8 137 215 1944

Neukomm S Un test sensible et ultra rapide du pouvoir cancerigene de certaines substances chimiques Oncologia *10* 107 119 1957

Newman M S & *Otsuka S* Rate and Equilibrium Constants for the Reaction of Maleic Anhydride with the Methyl 1,2 Benzanthracenes J Nat Cancer Inst *21* 721 728 1958

Pollia J A Investigations on the Possible Carconogenic Effect of Anthracene and Chrysene and some of Their Compounds—I The Effect of Painting on the Skin of Mice J Industr Hyg *21* 219 220 1939

Pollia J A and Chrysen • In jection

Irina C & *T* & Hartwell 1957)

Riegel B *Wartman W B* *Hill W T* *Reeb B B* *Shubik P* & *Stanger D W* Delay of Methylcholanthrene Skin Carcinogenesis in Mice by 1,2,5 6 Dibenzofluorene Cancer Res *11* 301 303 1951

Roe F J C & *Salaman M H* Further Studies on Incomplete Carcinogenesis Triethylene Melamine (T E M) 1,2 Benzanthracene and β Propriolactone as Initiators of Skin Tumour Formation in the Mouse Brit J Cancer 9 177 203 1955

Schmähl D Prufung von Naphtalin und Anthracen auf cancerogene Wirkung an Ratten Ztschr f Krebsforsch 60 697 710 1955

reviewed results in mammals was the negative reaction to 1 2 4 tri methylphenanthrene and the positive response to 3 ,6 dimethyl benz[a] anthracene The results, including some quantitative experiments are discussed, and it is preliminarily concluded that the newt test has shown sufficient specificity to be used as a quick test for carcinogenicity, at least of polycyclic hydrocarbons

REFERENCES

Andervont H B & Shimkin M B Biologic Testing of Carcinogens—II Pulmonary Tumor Induction Technique J Nat Cancer Inst *1* 225 239 1940

Arffmann F & Christensen B Collat- Studies on the Newt Test for Carcinogenicity —1 Benzo[a]pyrene Dibenz[a h]anthracene and 3 Methylcholanthrene Acta path et microbiol scandinav 52 330–342 1961

Arffmann F Studies on the Newt Test for Carcinogenicity —2 Tributyrin and Propylene Glycol as Solvents Acta path et microbiol scandinav 55 281 288 1962

Bachmann W F Cook J W Dansi A de Worms C G M Haslewood C A D Hewett C L & Robinson A M The Production of Cancer by Pure Hydrocarbons—IV Proc Roy Soc London ser B *123* 343 368 1937

Badger G M Cook J W Hewett C L Kennaway F L Kennaway N M & Martin R H The Production of Cancer by Pure Hydrocarbons—VI Proc Roy Soc London ser B *131* 170 182 1942

Badger G M The Carcinogenic Hydrocarbons Chemical Constitution and Carcinogenic Activity Brit J Cancer 2 309 350 1948

Badger G M Chemical Constitution and Carcinogenic Activity Advances Cancer Res 2 73–127 1954

Barry G & Cook J W A Comparison of the Action of Some Polycyclic Aromatic Hydrocarbons in Producing Tumours of Connective Tissue Am J Cancer *20* 58 69 1934

A D Hewett C L Hieger I & Kennaway E L Pure Hydrocarbons—III Proc Roy Soc London

Carcinogenesis—A Study of the Significance of Cocarcinogenic Action and Related Phenomena Cancer Res *1* 807 814 1941

Bergol ts V M & Il yina A A Biokhimiya *16* 262 268 1951 (Cit from Shubik P & Hartwell J L Survey of Compounds Which Have Been Tested for Carcinogenic Activity Supplement I PHS Publication No 149 U S Government Printing Office Washington 1957

Bottomley A C & Twort C C The Carcinogenicity of Chrysene and Oleic Acid Am J Cancer *21* 781 786 1934

Boyland F & Burrows H The Experimental Production of Sarcoma in Rats and Mice by a Colloidal Aqueous Solution of 1 2 5 6 D benzanthracene J Path & Bact *41* 231 238 1935

Chalvet O & Mason R Carcinogenicity of the Methyl 1 2 Benzanthracenes and their Derivatives Nature (Lond) *192* 1070 1072 1961

Cook J W Haslewood G A D Hewett C L Hieger I Kennaway F L & Mayneord W V Chemical Compounds as Carcinogenic Agents Rep IInd Int Congr against Cancer (Brussels) *1* 1 23 1936

Dannenberg H Beitrag zur krebserzeugenden Wirkung aromatischer Kohlenwasserstoffe und verwandter Verbindungen Ztschr f Krebsforsch *63* 102 117 1959

Domagk G Synthetische carcinogene Substanzen Medizin und Chemie 3 274 293 1936

Druckrey H & Schmahl D Cancerogene Wirkung von Anthracen Naturwiss *42* 159 160 1955

Dunning W F & Curtis M R Relative Carcinogenic Activity of Monomethyl Derivatives of Benz[a]anthracene in Fischer Line 344 Rats J Nat Cancer Inst 25 387 391, 1960

FROM THE INSTITUTE FOR GENERAL AND EXPERIMENTAL PATHOLOGY,
THE UNIVERSITY OF OSLO NORWAY

KINETICS OF EPIDERMAL CELL POPULATION OF THE HAIRLESS MOUSE

By

ØYVIND SKJÆGGESTAD

Received 16 x 62

The epidermis of hairless mice consists principally of one basal cell layer, a small varying amount of differentiating cells, and externally a horny layer (1) In studies of epidermal cell proliferation it is necessary to distinguish between the dividing process going on among the basal cells and the maturing process (keratinization) going on in the differentiating cells Thus the term "turnover time" cannot satisfactorily be applied to the epidermis as a whole We can, however, speak of a "mean generation time' for the basal cells exclusively, namely the average time period elapsing between two subsequent mitoses in the same cell The notion "turnover time" may be applied to the differentiating cells, indicating the time period elapsing from the formation of differentiating cell until it looses its nucleus and is transformed to "keratin"

At our institute we have found reason to believe that in the hairless mouse the "mean generation time" of the basal cells is about 5 days, and the "turnover time" of the differentiating cells around 3 days The "turnover time" of the horny layer is estimated at 1 3 days (1)

To ascertain, if possible, these statements, the extent and the time course of the cell loss from the epidermal surface after labelling epidermal cells once with tritiated thymidine has been studied If H³-thymidine is injected into an animal, this substance is exclusively taken up by the cells which are engaged in DNA-synthesis at the short time interval when thymidine is available in the blood Therefore, in the epidermis only some basal cells will be labelled After the first mitosis of a labelled cell, each of the two daughter cells contains half of the label Theoretically one of these cell
remains a basal ce
label remains in t
entiating cells Th ... activity of the tritium should remain at a constant value until the first differentiating cells and the horny

The author is indepted to *National Institute of Health Bethesda USA* who provided the spectrometer and to professor dr med *Lorentz Eldjarn* who put the instrument at my disposal

Schurch O & *Winterstein A* Über die krebserregende Wirkung aromatischer Kohlenwasserstoffe Hoppe Seylers Ztschr f physiol Chem *236* 79 91 1935
Shear M J & *Leiter J* Studies in Carcinogenesis — XVI Production of Subcutaneous Tumors in Mice by Miscellaneous Polycyclic Compounds J Nat Cancer Inst 2 241 258 1941
Steiner P E & *Falk H L* Summation and Inhibition Effects of Weak and Strong Carcinogenic Hydrocarbons 1 2 Benzanthracene Chrysene 1 2 5 6 D benzanthracene and 20 Methylcholanthrene Cancer Res *11* 56 63 1951
Steiner P E & *Edgcomb J H* Carcinogenicity of 1 2 Benzanthracene Cancer Res *12* 657 659 1952
Twort C C & *Fulton J D* Further Experiments on the Carcinogenicity of Synthetic Tars and Their Fractions J Path & Bact *33* 119 143 1930
White F R & *Eschenbrenner A B* Note on the Occurrence of Hepatomas in Rats Following the Ingestion of 1 2 Benzanthracene J Nat Cancer Inst *6* 19 21 1945

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cell each of the two daug[illegible]
[illegible] one of these cells be[illegible]
remains a basal cell When all the labelled cells have divided, half of the label remains in the basal layer, the other half is deposited in the differentiating cells Theoretically, the activity of the tritium should remain at a constant value until the first differentiating cells and the horny

The author is indepted to *National Institute of Health Bethesda USA* who provided the spectrometer and to professor dr med *Lorentz Eldjarn* who put the instrument at my disposal

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In practice, however, one cannot expect to observe a regular, steplike curve as mentioned above Biological variations and methodological errors will tend to smooth out the steps The activity can be expected to follow a curve characterized by a slight fall the first days, and then the curve may decrease exponentially Plotted on a semilogarithmic scale, the latter part of the curve will then become a straight line whose steepness is a function of the lenght of the "mean generation time' of the basal cell layer

For the purpose of examining the total radioactivity in the epidermis it is necessary to separate the epidermis from the rest of the skin with a method that gives a specimen of almost pure epidermis, and does not dilute the activity We have found the Castroviejo keratotome very useful for this purpose Using this method we succeeded in separating thin, even slices of epidermis from the rest of the skin Microscopical examination showed that very little of the corium adhered to the pieces of epidermis, and very few cells from epidermis remained in the corium

After weighing the epidermal specimens, they were homogenized with 1 ml 1 M methanolic solution of Hyamine in thightly capped vials and placed in a thermostate for 16 hours at 55° C After dissolving, 11 ml scintillation solution was added to the samples and these were cooled to the temperature of the Pakard Tri-Carb Spectrometer counting chamber (—10° C) Tritium was later counted at about 1150 volt and window setting 10 50 volt The reliability of this method has been checked and found satisfactory The total radioactivity per mg epidermis the first ten days after injection of 50 μc H^3-thymidine intraperitoneally to each mouse has been measured Table 1 and Figures 1 and 2 demonstrate the results

We suppose that the steep fall of the curve (Fig 1) from the third to the sixth day represent the period when cells from the "first generation" after labelling pass their "turnover time and are shed The average "turnover time" of the differentiating cells including the horny layer, when the DNA-synthesizing time, premitotic and mitotic phase are subtracted, can be set to about 4 days

From the steepness of the last part of the curve plotted on the semi-

TABLE 1

Mean Count per Min per mg the First Ten Days after one Single Intraperitoneal Injection of 50 µc H³–Thymidine

Days from injection	1/3	1	2	3	4	5	6	7	8	9	10
No of mice	16	46	16	20	16	16	18	16	16	16	16
Mean count per min per mg	223.9	205.8	191.6	182.9	146.5	131.5	100.0	101.7	83.9	69.8	68.1
SD of the mean	7.0	6.1	6.8	10.1	10.0	9.7	11.1	8.4	7.6	7.4	4.9

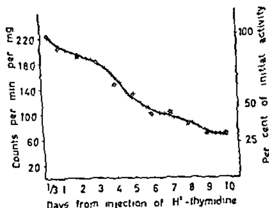


Fig 1

Total radioactivity of epidermis the first ten days after one single intraperitoneal injection of 50 µc H³ thymidine Dotted area 2 SD of the mean

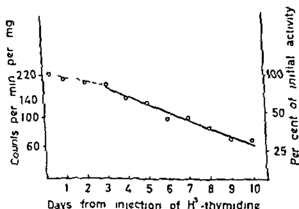


Fig 2

Total radioactivity of epidermis the first ten days after one single intraperitoneal injection of 50 µc H³ thymidine Plotted on semilogarithmic scale

logaritmic paper, Fig 2, (least squares method), the "mean generation time" of the basal cells can be calculated to cover about 5 days

The present results thus give a strong support to the previous calculations (1) which were based on studies of mitotic counts and mitotic

layer produced by the latter have past the "turnover time" At that time the activity can be expected to decrease to about 50 per cent of the initial activity, *e g* when all cells of the "first generation" have passed through the maturing process For the subsequent time period the activity should again be constant until the label which remain in the basal cells (50 per cent) has been further halved and the new differentiating cells of the "second generation "have passed their "turnover time' When all the latter are shed, the activity should be about 25 per cent of the initial value The reservation must be made, however, that this cannot be observed if the "mean generation time" of the basal cells is very different from the total "turnover time" of the differentiating cells and the horny layer combined (1)

In practice, however, one cannot expect to observe a regular, steplike curve as mentioned above Biological variations and methodological errors will tend to smooth out the steps The activity can be expected to follow a curve characterized by a slight fall the first days, and then the curve may decrease exponentially Plotted on a semilogarithmic scale, the latter part of the curve will then become a straight line whose steepness is a function of the lenght of the "mean generation time" of the basal cell layer

For the purpose of examining the total radioactivity in the epidermis it is necessary to separate the epidermis from the rest of the skin with a method that gives a specimen of almost pure epidermis, and does not dilute the activity We have found the Castroviejo keratotome very useful for this purpose Using this method we succeeded in separating thin, even slices of epidermis from the rest of the skin Microscopical examination showed that very little of the corium adhered to the pieces of epidermis, and very few cells from epidermis remained in the corium

After weighing the epidermal specimens, they were homogenized with 1 ml 1 M methanolic solution of Hyamine in thightly capped vials and placed in a thermostate for 16 hours at 55° C After dissolving, 14 ml scintillation solution was added to the samples and these were cooled to the temperature of the Pakard Tri-Carb Spectrometer counting chamber (—10° C) Tritium was later counted at about 1150 volt and window setting 10–50 volt The reliability of this method has been checked and found satisfactory The total radioactivity per mg epidermis the first ten days after injection of 50 μc H^3-thymidine intraperitoneally to each mouse has been measured Table 1 and Figures 1 and 2 demonstrate the results

We suppose that the steep fall of the curve (Fig 1) from the third to the sixth day represent the period when cells from the "first generation" after labelling pass their "turnover time and are shed The average "turnover time" of the differentiating cells including the horny layer, when the DNA-synthesizing time, premitotic and mitotic phase are subtracted, can be set to about 4 days

From the steepness of the last part of the curve plotted on the semi-

TABLE 1

Mean Count per Min per mg the First Ten Days after one Single Intraperitoneal Injection of 50 μc H^3–Thymidine

Days from injection	1/3	1	2	3	4	5	6	7	8	9	10
No of mice	16	16	16	20	16	16	18	16	16	16	16
Mean count per min per mg	223 9	205 8	191 6	182 9	146 5	131 5	100 0	101 7	83 9	69 8	68 1
SD of the mean	7 0	6 1	6 8	10 1	10 0	9 7	11 1	8 4	7 6	7 4	4 9

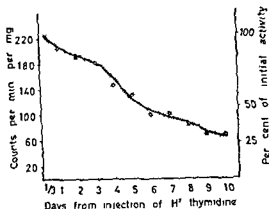


Fig 1

Total radioactivity of epidermis the first ten days after one single intraperitoneal injection of 50 μc H^3 thymidine Dotted area 2 SD of the mean

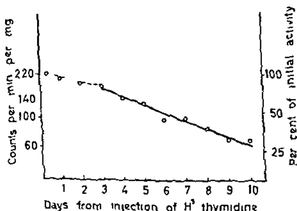


Fig 2

Total radioactivity of epidermis the first ten days after one single intraperitoneal injection of 50 μc H^3 thymidine Plotted on semilogarithmic scale

logarithmic paper, Fig 2, (least squares method), the "mean generation time" of the basal cells can be calculated to cover about 5 days

The present results thus give a strong support to the previous calculations (1) which were based on studies of mitotic counts and mitotic

rates Analogous results have been reported from studies on cell proliferation in bone marrow cells and in intestinal epithelium by *Steel* (2)

SUMMARY

The DNA synthesizing cells of the epidermis in hairless mice were labelled with tritiated thymidine The decrease of the radioactivity of the epidermis the following ten days was measured by a Liquid Scintillation Counter From the results obtained the "mean generation time of the basal cells was found to be about 5 days, and the 'turn over time of the differentiating cells and horny layer to be about 4 days These results give a strong support to previous calculations which were based on studies of mitotic counts and mitotic rates

REFERENCES

1 *Iversen O H & Evensen A* Experimental Skin Carcinogenesis in Mice Acta path et microbiol scandinav supplementum 156 1962
2 *Steel G G* Tritium in the Physical and Biological Science International Atomic Energy Agency Vienna 1962

FROM THE CENTRAL HOSPITAL IN RANDERS, DENMARK, THE LABORATORY OF PATHOLOGY (HEAD: CHIEF PATHOLOGIST J. V. THORBORG, M.D.)

A SPECIFIC, HISTOLOGICAL FLUORESCENCE STAIN FOR MUCOID SUBSTANCES WITH CONTENTS OF ACID MUCOPOLYSACCHARIDES

By

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Research work in recent years has introduced the more general use of fluorescence microscopy in routine work in histological laboratories. Interest has been focussed on the acridine orange stain which is used in screening of smears for tumour cells. To increasing degrees the more specific fluorescence stains are used in the field of tissue diagnostic, including also the special bacteriological stains. Using acridine orange, fluorescence is obtained mostly of nucleic acids and of acid mucopolysaccharides. The available literature on the nucleic acid stain is rather extensive whereas the acid mucopolysaccharide stain has been described less frequently, probably because the fluorescence here obtained is less marked.

In 1958 *Hicks & Matthaei* (3)—purely accidentally—were led on the track of a new method by which mucoid substances became stainable with acridine orange: a section already stained by the *Masson* trichrome method was further treated with acridine orange; on examination with ultraviolet light a brilliant fluorescence of the mucin in the epithelium was noted. This feature was analyzed and iron haematoxylin was found to be the reagent in the Masson method which was responsible for the reaction. Experimental series showed that other iron-containing solutions also induce a fluorescence selective for mucin; other generally applied mordants induce slight fluorescence only, if at all.

In routine microscopy with normally transmitted light a number of excellent methods are available by which to stain mucoid substances, the best known being PAS and alcianblue. Many substances—not infrequently substances the chemical structures of which are obscure—are PAS positive, but according to e.g. *Pearse* (5) such affinity is absent in the acid mucopolysaccharides. Alcianblue staining, as described by e.g. *Eskelund* (2) is combined with a nuclear staining. Alcianblue stains acid mucopolysaccharides; if used electively on frozen sections for a short time a pale bluish colour of the mucoid substances is obtained, but there is a certain staining of other tissue components. Hence,

at method seems to be required by which mucoid substances are readily stained selectively and therefore an attempt was made to modify the fluorescence method so as to make it applicable in the routine as a process by which frozen sections could be quickly stained

METHODS

The following technique has been suggested by *Hicks & Matthaei* for the staining of mucin and certain mucoid substances in tissue sections
Formalin fixation paraffin sections (or frozen sections)

1 Bring sections to water
2 Treat for 5 to 10 minutes by a 4 per cent solution of ferric alum
3 Rinse briefly in tap water
4 Stain for 2 minutes by an 0 1 per cent solution of acridine orange
5 Rinse briefly in tap water
6 Mount with glycerine
Examination with ultraviolet light using a yellow eyepiece filter
The fluorescence will modify the instability being less marked after treatment by ferric alum than after treatment by other iron containing solutions

The authors have examined various types of tissues and demonstrated a specific fluorescence of mucoid substances of different origins Hence the authors concluded that the acid mucopolysaccharides were responsible for the positive reaction

The following equipment has been used in the present study a Leitz Dialux *microscope with proportionate fluorescence equipment the source of* light being a Philips mercury lamp CS 150 W with heat filter and a BG 12 filter by which ultra violet blue light is obtained Copper sulphate solution was not introduced for the absorption of light from the red end of the spectrum as it was found to provide no noticeable effect A yellow eyepiece filter OG 1 was used

A series of staining processes were carried out according to the method suggested by *Hicks & Matthaei* in all essentials the results obtained conformed with the findings by these authors *Upon treatment by ferric alum all of the nuclear* fluorescence of the acridine orange disappeared the positive substances fluorescing a brilliant red Outlines were visible but only few details

In experimental series it was attempted to modify the fluorescence method so as to make it applicable as a method by which frozen sections might be quickly stained The attempt proved successful using the technique to be described later

RESULTS

The fluorescence observable in the tissue sections may be schematized as follows

Tissue	*Tissue component*	*Fluorescence*
Gastro intestinal tract	goblet cells	+++
	surface epithelium and glands	—
Mucoid intestinal carcinoma	mucoid substances	+++
Mamma	epithelium and glands	—
Fibro adenomatosis of the breast	epithelium of the cysts	(+)
Mucoid carcinoma of the breast	mucoid substances	+++
Secretory phase of endometrium	epithelium of the glands	(+)
Uterine cervix	surface epithelium and glands	+++
Polyp in uterine cervix	surface epithelium and epithelium of the glands and cysts	+++
Mucinous cystadenoma of ovary	epithelium of the cysts	+++
Parotis	epithelium of the glands	
Mixed tumour of the parotid gland	ground substance	++
Cartilage	ground substance	+++
Osteochondrosarcoma	ground substance	+++

In the gastro intestinal tract goblet cells are seen to fluorescence strongly, surface epithelium and glands, including the duodenal Brunner's glands, emit no fluorescence, in the colon a positive layer of mucus is not infrequently seen on the surface epithelium In highly dilated goblet cells the fluorescence will be least pronounced centrally in the cells, probably because the mucin here has been eliminated during preparation, indeed, it may be ascribable to variations in the chemical composition of the mucin centrally and peripherally in the cell A strong, positive reaction is given by mucoid, intestinal carcinomata Reactions are negative of normal glands of the breast, in fibroadenomatosis the epithelium of the cyst becomes faintly stained, the mucoid substances in carcinomata of the breast give strong fluorescence During the secretory stage the glandular epithelium of the endometrium may become faintly stained, the secretion in the lumen of the glands is seen to emit a stronger fluorescence In the uterine cervix the secretion matter as well as the glands and the surface epithelium are seen to give a strong, positive reaction The same is noted in polyps in the uterine cervix and in mucinous cystadenomata of the ovary The parotid gland gives negative reaction Some of the mixed tumours in the parotid gland contain large areas in which reactions of the ground substance are positive Cartilaginous ground substance and the ground substance in osteochondrosarcomata give positive reactions

Using chromium reagents I have tried to obtain a similar quenching of the acridine orange fluorescence of tissue components containing nucleic acid, on the consideration that chromium and ferric reagents have several chemical properties in common Reactions obtainable by chromium were small, however, and the medium must be considered inadequate for the purpose

On the basis of experimental series the fluorescence method has been modified to be a fast method for the staining of fresh, non-fixed, frozen sections

(1) Treat for 30 seconds by a 10 per cent solution of ferric alum
(2) Rinse briefly in tap water
(3) Stain for 60 seconds by a 0 1 per cent solution of acridine orange
(4) Rinse briefly in tap water
(5) Mount immediately with glycerine

Upon preparation specimens must be examined without delay with ultraviolet light since fluorescence soon will be fading to a certain degree this feature, however, is rather immaterial here, the purpose being to obtain a method for frozen sections to be examined immediately The staining intervals recorded above should be taken as minimums they cannot be further reduced but, time permitting, it may prove an advantage to use longer staining intervals

DISCUSSION

Previous investigators have classified mucopolysaccharides according to different patterns Mucopolysaccharides are proteins containing a prosthetic group built up by nitrogenous heteropolysaccharides (1) which constitute a considerable part of the molecule, generally more than 5 per cent Mucopolysaccharides are divided into two groups the acid and the neutral ones, the former being highly labile are isolated only with difficulty, the latter presenting solid linkage between the protein and the prosthetic group Using spectral curves from fluorescence stained substrates *Kuyper* (4) was successful in identifying the various mucopolysaccharides This author preferred coriphosphine to acridine orange finding the colour differences of the various substances to be more distinct after treatment with the former

Acid mucopolysaccharides are assumed to be responsible for the fluorescence in the *Hicks and Matthaei* stain and this theory has been substantiated by *Kuyper*'s findings (4) as well as by the components observed in the treated tissues Besides, since acid mucopolysaccharides are very labile in the presence of alkaline a number of control sections were treated prior to staining using S P L Sorensen's phosphate buffer, pH 8 6, followed by rinsing in tap water ,the result of which was a blurring or a considerable reduction of the fluorescence

For diagnostic purposes tumour cells are generally stained by an 0 01 per cent acridine orange solution Cells would become overstained if an 0 1 per cent solution were used In the staining process of mucoid substances a treatment by iron operates to inhibit the normally produced acridine orange fluorescence in almost all of the tissue components with the exception of mucin and certain mucoid substances, the normal fluorescence of which is intensified at the high concentration of dye Treatment by iron-containing substances with a view to inhibiting undesired fluorescence has more recently been successfully exploited in other specific fluorescence staining processes, *e g* by *Vassar & Culling* (6, 7)

Staining according to the method suggested by *Hicks & Matthaei* has been used for paraffine sections and frozen sections of fresh or formalin fixed tissues Results were found to be superior if frozen sections of fresh or formalin fixed tissue were used The fluorescence obtained in paraffine sections might be rather uneven, the reason why being obscure the elimination of the acid mucopolysaccharides in the many baths may play a certain rôle but chemical changes of the acid mucopolysaccharides in the baths or in the warm paraffine cannot be precluded

CONCLUSION

The fluorescence stain for mucoid substances as described by *Hicks & Matthaei* is of the greatest, theoretical interest but in the field of microscopy by normally transmitted light many valuable stains are

available for the same substances and it can hardly be doubted that the latter will have preference in practical routine work, mostly because the fluorescence is prone to disappear But, a method by which frozen sections can be quickly stained is an urgent demand With a view to meeting such demand the present paper presents a modification of the fluorescence method Drawbacks involved in the fluorescence technique are but small always provided that the equipment is permanently established in connection with the generally used microscope

SUMMARY

Hicks & Matthaei used acridine orange for the staining of mucoid substances considering it a specific histological stain in which the fluorescence of components containing nucleic acids was quenched by treatment with ferric alum On the basis of personal studies this method is discussed in the present paper The fluorescence obtained in the various tissues is described and the theory that acid mucopolysaccharides may be responsible for the fluorescence is strongly substantiated With a view to covering the demand for a quick staining method applicable to mucoid substances in frozen sections the author has introduced a modification of the fluorescence method

REFERENCES

1 *Cantarow A & Schepart B* Biochemistry Saunders Philadelphia & London 1957
2 *Eskelund V* Mucin staining with alcian blue Acta path et microbiol scandinav 40 107 109 1957
3 *Hicks J D & Matthaei F* A selective fluorescence stain for mucin J Path & Bact 75 473 476 1958
4 *Kuyper Ch M A* Identification of muc polysaccharides by means of fluorescent basic dyes Exptl Cell Research *13* 198–200 1957
5 *Pearse A G E* Histochemistry Churchill Ltd London 1960
6 *Vassar P S & Culling C F A* Fluorescent stains with special reference to amyloid and connective tissues Arch Path 68 487 498 1959
7 *Vassar P S & Culling C F A* Fluorescent amyloid staining of casts in myeloma nephrosis Arch Path 73 59 63 1962

FROM THE RHEUMATIC RESEARCH LABORATORY, UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY (CHIEF G TEILUM, M D), COPENHAGEN, DENMARK

ELECTRON-MICROSCOPIC APPEARANCE OF THE MYOMETRIUM OF CERVIX UTERI IN CASTRATED GUINEA PIGS TREATED WITH SEX HORMONES

By

O H IVERSEN and H E CHRISTENSEN

Received 5 xi 62

In a previous publication *Iversen* (6) described the histologic and histochemical changes in the interstitial connective tissue of the myometrium in castrated guinea pigs after treatment with oestrogen and androgen hormones It was concluded that the amount of collagen was increased following oestrogen and androgen administration, and at the same time a widening of the interstitia between the muscle cells was found The latter phenomenon was possibly due to an increased amount of ground substance

The present electron-microscopic investigations are a direct continuation of the above mentioned studies as an attempt to confirm the statements at the ultrastructural level Only the changes seen in the myometrium of the cervix uteri are described in this paper

MATERIAL AND METHODS

Young female guinea pigs weighing 600 700 gms were employed
The animals were divided in 5 groups

Group 1 Untreated normal animals
Group 2 Oophorectomized control animals
Group 3 Oophorectomized animals treated with 0 25 mgs oestradiol dipropionate (Diprovex Leo ® intramuscularly) every second day Total number of injections 4
Group 4 Oophorectomized animals treated with 0 5 mg of oestradiol dipropionate (Diprovex Leo ® intramuscularly) daily for 14 days
Group 5 Oophorectomized animals treated with 0 5 mg of testosterone propionate every second day Total number of injections 4

The oophorectomia was done in intraperitoneal Nembutal ® (Abott) anaesthesia
xtirpation of the ovaries
inhalation The hormonetreated ones were
mmediately after the death of the animal
mall tissue blocks measuring about 1 cmm
were cut from the myometrium of the cervix with a razor blade and promptly fixed for two hours in buffered osmium tetroxide (10) After fixation they were de

Aided by grants from the *Danish League against Rheumatism* and the *Danish State Science Foundation*



Fig. 1

Gr up / ... inters ... en b ... by na ... the c ... plasmic protrusion. The *interstitial* spaces are narrow and contain densely arranged collagen × 18000

hydrated in acetone and embedded in Vestopal (12). Thin sections were cut on a LKB ultramicrotome. The sections were after stained for two hours in 0.5 per cent uranyl acetate. For the electron microscopy a RCA (EMU 2E) electron microscope was used.

The ... from ... my m ... 4th ...

RESULTS

In evaluating the findings obtained by electron-microscopy of the myometrium of the cervix uteri from guinea pigs given the above-mentioned treatment in the five groups it seems difficult to decide.



Fig 2

Group 1—normal animal The intercellular space between two muscle cells is packed with dense collagen in a scanty matrix or ground substance The vacuoles just beneath the cell surface of the muscle cell represent a common finding (compare Fig 1) × 18000

whether a given change was due to the treatment or for instance a deviation from the normal state Therefore only the changes found in more than one series of sections are mentioned or illustrated as representative for the respective group The results were as follows

Group 1 Untreated normal guinea pigs As already known from ordinary histology the smooth muscle cells are closely packed rather narrow and elongated The interstitia are narrow slits with few interstitial cells and collagen if present is found in dense boundles By electron microscopy an additional observation was that the surface of the muscle cells often possessed flabby protrusions interdigitating with those from an adjacent muscle cell across the narrow interstitial space Fig 1 shows the normal muscle cells with the myofibrils running parallel to the nucleus and the cell surface in a rather narrow zone Rather

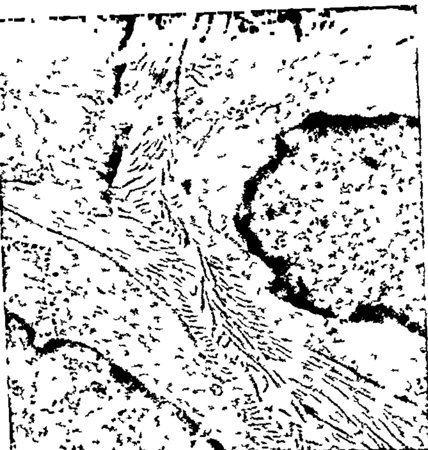


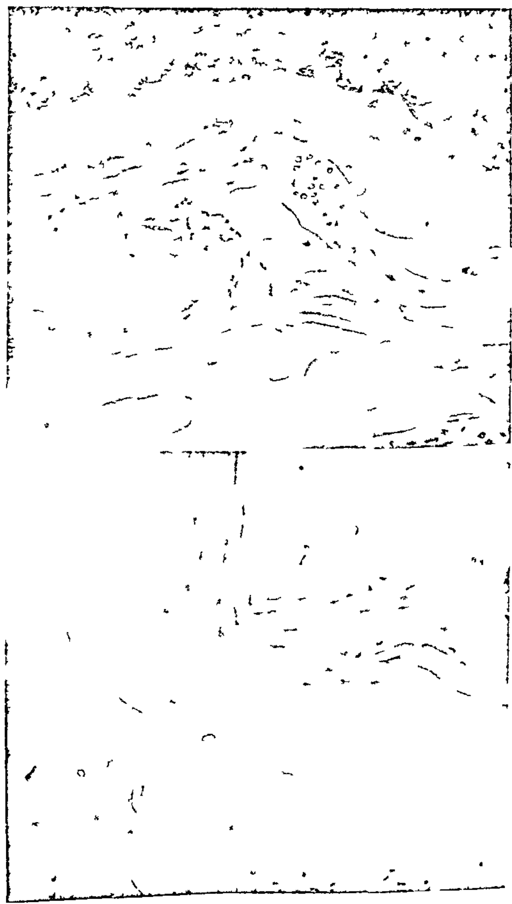
Fig 3

Group 2—castrated animal The cytoplasm of the three muscular cells show atrophic changes especially in the zone of myofibrils The surfaces of the muscular cells are rather smooth The interstitium is not very wide (as in groups 3 5 Figs 5–8 and 10 but contains much collagen possibly more loosely arranged than in group 1 (Figs 1 and 2) × 40000

densely packed collagen is present in the interstitia Fig 2 illustrates an interstitium between two muscle cells filled with dense collagen

Group 2 Oophorectomized control animals The general impression was that the smooth muscle cells showed atrophy The myofibrils had not the distinct appearance noted in group 1 and the different zones in the cytoplasm were not clearly separated Collagen was present in a rather large amount in the interstitia (fig 3)

Group 3 Oophorectomized guinea pigs treated with low estrogen dosage The amount of collagen in the interstitia between the muscle cells ap



peared to have increased as compared with groups 1 and 2, and collagen was found in all interstitia, being spread more diffusely rather than collected in dense bundles It was evident, that the interstitia had widened, and as the collagen fibrils seemed to be separated from each other, it had to be considered whether more interfibrillar matrix was involved as compared with the previous two groups This matrix may be oedema or rather be of the character of normal ground substance Fig 4 illustrates the condition in an animal from this group Portions of two interstitial cells are seen They contain large mitochondries and also a a rather well developed ergastoplasm In addition an inclusion body is seen in one of the cells, this may be identical with a small Kurloff body (2), admittedly produced in castrated guinea pigs after a estrogen administration, and occasionally visible by light microscopy in PAS-stained preparations Fig 5 illustrates the rather loose arrangement of the collagen in the widened interstitium

Group 4 Oophorectomized guinea pigs treated with high oestrogen dosage This was the group of animals, which showed the most pronounced changes from the normal picture Wavy bundles of collagen were present in large amounts The interstitia had widened considerably The muscle cells showed vacuolation and loss of structure The nuclei were large and less osmiophilic than normally, often they had a vesicular appearance (figs 6 and 7)

Group 5 Oophorectomized guinea pigs treated with androgen After treatment with testoteron propionate an increase of collagen was seen This was in quantity and type of arrangement midway between the findings in oestrogen treated and normal animals (figs 8–10) In addition inclusion bodies were found in some muscle cells (figs 9 and 10), these were different from the inclusions of Kurloff body type, although these also may be produced in castrated guinea pigs by means of androgen substance In Fig 9 the inclusion body is attached to the periphery of a mitochondrion

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Figs 4 5

Fig 4 Group 3—oestrogen treated castrated animal Uppermost is a portion of a smooth muscle cell with nucleus double nuclear membrane narrow cytoplasmic zone and cell surface Then follows the wide interstitial space containing *collagen and traversed by two interstitial cells* The collagen shows periodicity or transversally cut it appears ring shaped and the individual fibrils lie in a [illegible]
granular of fib[illegible]
loped ergastopl[illegible]
× 40000

Fig 5 Gro[illegible]

DISCUSSION

The effects of oestrogen hormones on the mammalian uterus have been described by several investigators *Reynolds* (9) summarized these to be hyperaemia, water retention, increased cell volumina and general metabolic changes *Leathem & Wolf* (8) investigated the effect of one single dose of 88 mikrograms oestrogen on immature guinea pig uteri and observed an increase of the weight to the double value *Velardo* (11) reported the weight-increasing effect of different estrogen substances, finding oestradiol to be the most powerful The quantitative data showed correspondance as regards wet and dry weights percentage of water and nitrogen content Testosteron induced changes were furthermore studied, and doses of 2–5 mg for 3 days did result in the same weight increase as 0,10 mg oestradiol 17 β *Frieden, Steele & Telfer* (3) observed an increased synthesis of collagen in the uterus following oestrogen stimulation The time relation between oestrogen administration and collagen amount in uteri of immature rats was investigated by *Hurley & Heermann* (5) After one single intraperitoneal injection of 0,1 microgram oestradiol no increase of collagen was noticed after 24 hours, but after three days the weight had increased four times and the collagen increased to the double No changes were found in skeletal and heart muscles

Harkness, Harkness & Moralee (4) found a collagen increase in uteri from castrated rats after oestrogen treatment on the basis of hydroxyproline determinations But the increase was of relatively late occurrence and quantitatively much less pronounced than the growth of the uterus as a whole (weight, N-content), so that the concentration of collagen indeed was decreased No difference in types of reaction was found between the cervix and the uterine horns

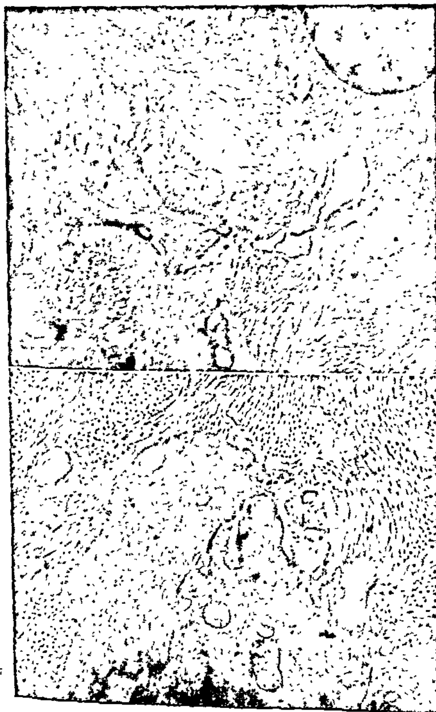
Iversen (6) using histochemical methods found evidence for mobilization of the ground substance and increased collagen content in the uteri of castracted female guinea pigs after oestrogen as well as androgen treatment The present electron-microscopic investigations also point to a definite increase of collagen in the myometrium of cervix uteri in oophorectomized guinea pigs following oestrogen administration At the same time a structural change in the appearance of the collagen was noticed, so that the collagen fibrils are more diffusely spread and more separated from each other, which arrangement is in contrast to

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Figs 6 7

Fig 6 Group 4—animal treated heavily with oestrogen Wavy bundles of collagen are seen to run in all directions Large vacuoles are present in cytoplasmic cell protrusions The nucleus in the upper right corner is somewhat vesicular × 27500

Fig 7 Group 4—animal treated heavily with oestrogen The interstitial spaces are rich in collagen and in interfibrillar matrix, which is some places is of fluffy appearance × 48500


6
7


8
9
10

Figs 8 10

Fig 8 Group 5—testosteron treated castrated animal The interstitial space between two muscle cells contain collagen of rather loose arrangement and a considerable amount of interfibrillar matrix × 18000

Fig 9 Group 5— testosteron treated castrated animal An inclusion body situated on the outer membrane of a mitochondrion in addition to vacuolization are seen An interstitial space of the same character as the one in Fig 8 separates these cellular constituents from the muscle cell in the upper right corner × 18000

Fig 10 Group 5—testosteron treated castrated animal The interstitium is similar to the one in Fig 8 but in addition portions of interstitial cells are present Within the smooth muscle cell below an inclusion body is seen × 18000

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the findings of the sporadic dense bundles of collagen in the normal animals This change in structure after oestrogen stimulation may be due to new formation of collagen and simultaneously increased production of interstitial ground substance or simply tissue oedema These phenomena may explain the increased widths of the interstitia between the muscle cells The findings of large interstitial cells with well-developed ergastoplasm (figs 4 and 5) are in accordance with the assumption of an increased synthesis of the mucoproteins, mucopolysaccharides and fibrils of that particular connective tissue Some of these cells undoubtedly are fibroblasts However, others may be part of the until now unexplained reaction seen in the reticular tissue of guinea pigs after oestrogen treatment preceding the formation of Kurloff bodies (2)

Asadi, Dougherty & Cochran (1) in electron microscopic investigations of loose subcutaneous connective tissue found that polymerized ground substance by virtue of its property of macro-molecules appeared granulated Although we sometimes, especially in the animals treated with heavy doses of oestrogen, found "cloudy" masses between the collagen bundles, our experiments do not show anything definitely about the state of polymerization of the ground substance

The treatment with androgen substance seems to have a similar effect on the uteri of the oophorectomized guinea pigs as the oestrogen treatment, but the changes are less pronounced This is especially seen in the increase of the interstitial spaces—an observation which is in accordance with previous histochemical investigations by *Iversen* (6) The appearance of the collagen arrangement is as in the oestrogen-treated animals, but quantitatively the total amount of collagen seems lesser

Our experiments are in accordance with the above mentioned investigations of *Harkness* et al with regard to an increase in the absolute amount of collagen during hormonal treatment But in addition we found that the collagen becomes arranged in a more loose-structured framework in broader interstitia with increased amount of ground substance (and/or oedema)

Hence, although we have a lower concentration of collagen per square unit the much increased width of the interstitia explains that the total amount of collagen is increased

Furthermore we found signs of increased synthetic activity in many interstitial cells namely the higher degree of organization of the cytoplasm which findings may be the cellular substrate for the changes in the interstitia

SUMMARY

The connective tissue changes in the myometrium of cervix uteri in oophorectomized guinea pigs after treatment with oestrogen and androgen hormones have been investigated by electron microscopy The main findings were that hormonal treatment causes increase in the total amount of collagen, partly due to a much increased width of the interstitia between he muscle cells At the same time the structural arrangement of collagen was altered the fibrils being more separated from each other lying in a more abundant matrix or ground substance The interstitial cells also showed signs of increased synthetic activity Treatment with oestrogen hormone gave the most pronounced effect The findings are discussed in relation to other previous works on the subject

REFERENCES

1 *Asadi A M Dougherty T F & Cochran G W* An Electron Microscopic Study of the Ground Substance of Connective Tissue Nature *178* 1061 66 1956

2 ... the Cytochemistry ... a Pig Reports from ... openhagen 196?

3 ... on the incorporation of labeled Amino Acids in Tissues of the Reproductive Tract Fed Proc *11* 215 1952

4 ... *alee B E* The Effect of oestrus Cycle ... Content of the Uterus of the Rat J

5 ... Estrogen and Progesteron on Collagen ... keletal Muscle Fed Proc *14* 230 1955

6 *Iversen O H* The influence of estrogenic and androgenic Hormones on the Mast Cells and Connective Tissue in Uterus of Guinea Pig Acta path et microbiol scandinav *56* 245 252 1963

7 *Iversen O H & Christensen H F* Light and Electron microscopic Studies on Connective Tissue Changes in the Myometrium of the Pregnant Guinea Pig To be published Acta path et microbiol scandinav

8 *Leathem J H & Wolf R C* The ... Mammals ... Physiology of Reproduction

9 *Reynolds S R M* Hormonic and ... Cold Spring Harbor Symposia on quantitative Biology Vol 5 84 ... 1937

10 *Palade G E* Study of Fixation for Electron Microscopy J Exp med *95* 285–298 1952

11 *Velardo J T* Steroid Hormones and Uterine Growth Annals of the New York Academy of Science 75 2 441–460 1959

12 *Zelickson A S & Hartman J F* Vestopal W A superior imbedding Medium for an Electron Microscopic Study of human Epidermis The journal of invest dermat *35* 315–317 1960

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LIGHT- AND ELECTRON-MICROSCOPIC INVESTIGATIONS OF THE UTERINE CONNECTIVE TISSUE IN PREGNANT GUINEA PIGS

By

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Received 5 xi 62

The changes occurring in the uterus during pregnancy as seen by ordinary histological methods are fairly well known This is especially true for the epithelial and stromal reactions of the endometrium owing to the large proportion, which this tissue constitutes of the samples in surgical pathology Less often the myometrium and uterine connective tissue in pregnancy have been the subject of morphological studies Keiffer 1923 (13) described the conditions of the uterine wall of lesser mammals at term and found a relative increase of intercellular sub stance, fibrils and capillaries *Stieve* 1932 (25) observed hypertrophy as well as proliferation of the smooth muscle cells in the human pregnant uterus *Krichesky* 1942 (14) found a considerable oedema of the stromal tissue just prior to delivery

Many of the new methods employed in connective tissue research of recent years have also been used in investigations of that tissue in the female genital organs during pregnancy Especially the changes in water content collagen and other connective tissue fibrils, ground sub stance and cellular elements in the connective tissue of the uterine wall have been investigated (*Harkness & Harkness* (6), *Maibenco* (15), *Monfort & Perez Tamayo* (17), among others)

The present paper reports on a combined study comprising histo chemical investigations by light microscopy and ultrastructural studies of uteri of young pregnant guinea pigs A correlation has been made between earlier results of biochemical, morphological and histochemical character and the findings reported herein

MATERIAL AND METHODS

10 young female guinea pigs weighing 400 500 gms were copulated with males and pregnancy was verified by explorative laparotomy 5 of these animals were killed by ether inhalation about the middle of the pregnancy period, while the remaining 5 animals were killed in the same way immediately after delivery

Aided by grants from the *Danish League against Rheumatism* and the *Danish State Science Foundation*

5 normal uncopulated female guinea pigs of same age and weights served as control animals

At autopsy the uterus was carefully removed *in toto* and small tissue pieces were cut off with razor blades for fixation to electron microscopy and light microscopy respectively The tissue was taken both from the cervix and the pregnant horn(s) for electron microscopy it was cautiously endeavoured to fix only myometrium

For *electron microscopy* tissue blocks measuring about 1 cubic millimetre immediately were fixed in buffered osmium tetroxide (20) for 1 to 2 hours at 4° C They were then embedded either in n butyl metachrylate (19) or in Vestopal W (26) Thin sections were cut in a LKS ultramicrotome Some of the metachrylate embedded sections were after stained in a 1 per cent solution of phosphotungstic acid for 1 hour (22), while Vestopal embedded sections always were after stained in a 0 5 per cent solution of uranyl acetate for 2 hours (22) The sections were examined in a RCA EMU 2B electron microscope

For *light microscopy* the tissue was fixed for 24 hours in neutral formalin 4 per cent and in a 4 per cent solution of lead subacetate Dehydration was done in ethanol of successive increasing concentration and xylole After embedding in paraffin sections were cut 5 micra thick

The *staining reactions* employed were

1 *Formalin fixed preparations* Haematoxylin eosin van Gieson method Mallory s connective tissue stain silver impregnation for reticulin periodic acid Schiff reaction (PAS) (21) elastin staining (5) methylgreen pyronin a m Unna Pappenheim (21) silver reaction for metalophil reticular cells (16) Hale technique with and without additional PAS reaction (18) alcian blue staining alone or in combination with safranin or azur A (24) and astra blue staining (2)

2 *Lead subacetate fixed specimens* Toluidine blue 0 1 per cent solution in buffer pH 4 5 for 30 min Dehydration in ethanol

In addition *histo enzymatic treatment* was used prior to staining in order to improve the specificity of the staining reactions Streptococcal hyaluronidasis (kindly supplied by V *Faber* M D The State Serum Institute Copenhagen) and

Hale reactions and the ribonuclease treated with Unna Pappenheim stain

RESULTS

By *light microscopy* it was seen that the endometrium of the corpus uteri was broader than that of the cervix This was especially the case towards the end of the pregnancy As mentioned above little attention has been paid to the epithelial changes as this study was particularly a connective tissue study It only shall be noted that the mucinous glands of the cervix often extended to the myometrium and were filled with secretion material Besides slight variations the connective tissue of the endometrium exhibits the same changes as in the myometrium

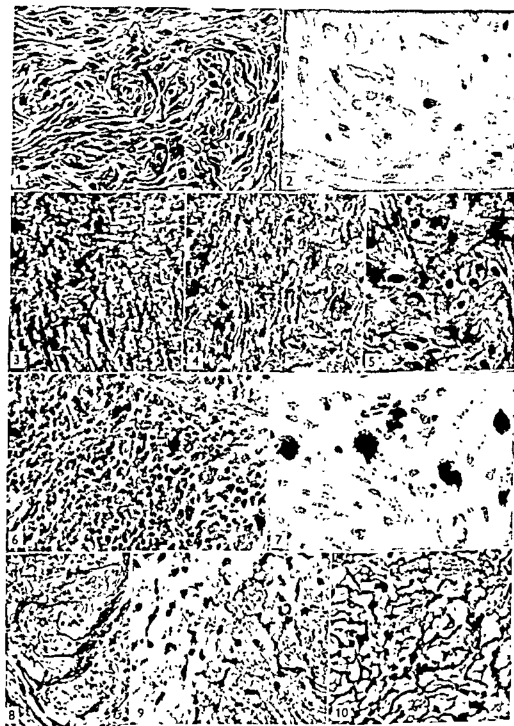
In the pregnant animals the myometrium was largely hypertrophied in comparison to the control animals The muscle cells had increased the volumes both of the nuclei and the cytoplasm At the end of the pregnancy period the cytoplasm showed a heavily positive PAS reaction As this reaction could be inhibited by pretreatment of sections with diastase, it is indicative for the presence of glycogen in the muscle cells In comparison with the normal animals the interstitial tissue spaces were much increased in width and contained much collagen By the van Gieson method and Mallory s connective tissue stain it was visualized, that this comes through a gradual increase of the total

amount of collagen during the whole pregnancy period Especially the corpus showed this reaction pattern while the cervix seemed to exhibit a lesser marked increase of collagen It is characteristic for the collagen in the pregnant animal to have more loose but wide spread arrangement In the normal animal the uterine collagen was found in bundles and streaks of firm texture and high stainability (Fig 1)—this is in contrast to the diffuse localization observed in the pregnant ones (Fig 2) Owing to the hypertrophy of muscle cells and of interstitial spaces the closeness of cells seemed lesser marked in the pregnant than in the normal animals

The histochemical reactions of the ground substance present in the intercellular and interfibrillar spaces tended to show a mobilization of that substance in pregnancy In the endometrium the Hale reaction was constantly positive In the myometrium the interstitial spaces contained a substance showing less pronounced Hale positivity than in the endometrium but on the other hand much exceeding that of the normal controls which may be negative No difference between corpus and cervix was observed in this respect Parallel to the increase in amount there was an increase in Hale positivity of the myometrial ground substance just at the end of pregnancy Alcian blue and astra blue stainings showed a reaction pattern corresponding to that of the Hale reaction but taken as a whole there was less coloration of the ground substance but here also this coloration far exceeded the results obtained with the normal controls The ground substance did not exhibit metachromasia with toluidine blue or azur A with the fixations and pH s used here unless in the very neighbourhood of mast cells (see below) The PAS reaction was constantly positive for the ground substance in the endometrium as well as in the myometrium and more positive than in normals But the PAS positivity might be fibrillar and indeed it was difficult to evaluate if such staining arose from staining of reticulin of young collagen Occasionally reticular cells with PAS positive granules were seen Kurloff bodies were found in few cells (4) Especially towards the end of pregnancy some small vessels of the myometrium showed an intima which was heavily PAS stained Elastin staining was constantly negative both in endometrium and myometrium

Reticulin fibrils were scanty in the normal control animals *whereas* they were present in great numbers in the pregnant animals both in endometrium and myometrium But perhaps some silver staining as the above *mentioned* PAS staining might be due to condensed ground substance Towards the end of the pregnancy the reticulin became thicker and of a more brownish colour and most probably in that state it is identical with collagen (Figs 3 4 5)

The impregnation for metalophil *reticular* cells showed a significant rise in number and also increase in size of cell body in the pregnant uterus (Fig 8 9 10)



Microphotographs from myometrium of cervix uteri.

Figs 1 10

Fig 1 Normal control animal Dense and heavily stained collagen in the interstitia between the muscle cells v Gieson method × 550

Fig 2 At term The hypertrophic muscle cells are illustrated Between them inter

Fig 3

Fig 4 Fourth week of pregnancy The spiral formed reticulin as in Fig 3 is not seen in this stage, nor is the brown stained thick collagen visible But some walls of small vessels and intermuscular spaces stain as reticulin Reticulin stain × 550

Fig 5 At term The hypertrophic muscle cells are separated by fibrillar masses staining partly as reticulin and partly as collagen Reticulin stain × 550

Fig 6 Normal control animal Several mast cells are shown together with the closely packed muscular cells of the resting organ The mast cells were crowded with numerous granules Compare Fig 7 Toluidine blue staining on lead subacetate fixed specimen × 550

Fig 7 At term As in Figs 2 and 5 hypertrophic muscle cells are present but only the nuclei of these are seen Mast cells are enormously enlarged and were surrounded by a metachromatic halo which is badly visible in the black white reproduction However the mast cell granules are less closely packed than in the smaller mast cells shown in Fig 6 Toluidine blue staining on lead subacetate fixed specimen × 550

Fig 8 Normal control animal As in Figs 1 3 and 6 all cell types appear smaller than in the pregnant animals Only few small metalophil reticular cells are present in the interstitia In addition some of the collagen bundles have been impregnated with this silver method Marshall impregnation for

Fig 9

Fig 10 ... in comparison with Figs 8 and 9 the metalophil reticular cells have further increased in number and size They apparently have become more branched and are anastomosing with each other Marshall impregnation for metalophil reticular cells × 220

The mast cells of the uterus were the cells, which showed the most pronounced changes during the course of pregnancy They were most numerous in the interstitial tissue of the myometrium, especially in its outer half, but they were also found perivascular In the basal layers of the endometrium they were found in smaller quantities The number of cells in the middle of pregnancy was nearly as in normal controls, but at the end of the pregnancy a considerable increase in number was observed This was especially the case in the cervix, where the mast cells during the whole period of pregnancy exhibited the greatest number The morphology of the cells changed at the same time At the beginning the cells were rather large and containing numerous well-defined granules stained deeply blue-violet with azur A and toluidine blue (especially in lead subacetate-fixed preparations) (Fig 6) But at the end of the pregnancy the mast cells are larger, and most of them are surrounded by a metachromatic halo, whereas the granules are less distinctly seen, and in some cells vacuoles are found (Fig 7) These changes were seen both in the corpus and in the cervix mast cells In



Fig 11

Electron micrograph The distribution of collagen in the myometrium of cervix uteri after 3-4 weeks of pregnancy The collagen in the interstitia is more diffusely arranged and less dense than in normal controls The muscle cells running obliquely in the section show hypertrophy and vacuolization Metachrylate embedding and after staining with phosphotungstic acid demonstrate clearly the collagen in black colour It is seen as black dots when transversally cut × 10500

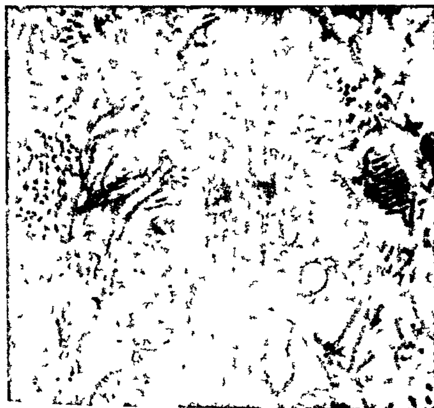


Fig 12

El tron m rog aph H gher magn f cat on of an area c rre pond ng to Fig 1 A part of a long tud nall cut muscle s seen n the m ddle w th nterst tial spaces on b th s des and to the left n add t on pa t of another muscle cell The ultra s u tural appearance of the collagen s v sual ze l and period t may be seen mate relat onsh p bet veen some k nd of attachment h phosphotungst c ac d

formalin fixed sections some mast cells stained heavily with alcian blue and astra l lue l ut not with safranin

The mast cells were not to l e foun l again after incul at on with strep tococcal an l testicular hyaluronidases and subsequent staining with tolui l ne l lue However just the same reaction was seen after incuba t on with the McIlvaine l uffer alone Thus the disappearance of the mast cell granules and the metachromatic halo is not necessarily a specif c action of the hyaluronidases

In the methylgreen pyronin staining a m Unna Pappenheim some mast cells contained orange granules i e reacted metachromatically with pyron n Somet mes an additional red st uning of the cytoplasm was ol serve l and this was removal le w th ril onuclease

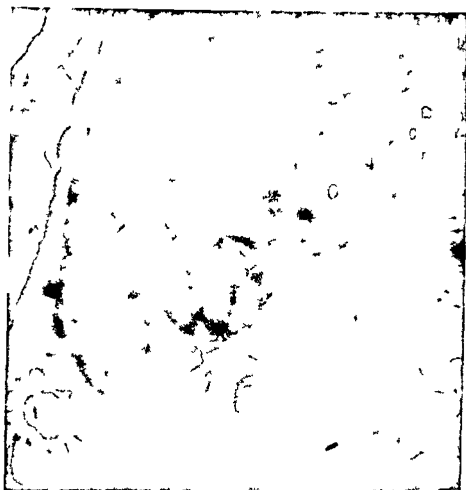


Fig 13

Electron micrograph A portion of a smooth muscle cell and an interstitial space with loosely arranged collagen in an abundant matrix (or edema) Specimen from animal just after delivery Vestopal embedded and uranyl acetate stained × 18000

By *electron microscopy* only the myometrium was thoroughly investigated In normal animals the smooth muscle cells were in close contact with each other as the intercellular spaces were narrow slits and when collagen was present it was arranged in dense bundles (Fig 1 and 2) In the pregnant animals the ultrastructural picture was quite different The muscle cells were larger in all dimensions Fig 11 shows the condition in an animal after 3 4 weeks of pregnancy The hypertrophied smooth muscle cells run parallel to each other but are separated by rather wide interstitia containing much collagen predominantly of loose texture and lying in a considerable amount of ground substance The black appearance of the collagen is due to afterstaining with phosphotungstic acid The vacuolization of the muscle cells represented a common finding which however may be fixation artefacts

The intimate relationship between collagen threads and the muscle



Fig 14

Electron micrograph An interstitial space between two muscle cells just after delivery Collagen is present in fairly rich amounts but the distance between the individual fibrils is larger than in normal animals (12 Figs 1 and 2) and in the earlier stages of pregnancy (Figs 11 and 12) Vestopal embedded specimen after stained with uranyl acetate × 27500

cells is illustrated in Fig 12 Immediately after delivery very wide interstitia were seen with an abundance of collagen fibrils lying in an ample matrix (Fig 13 and 14)

DISCUSSION

The present investigations, besides the well-known hypertrophy of the smooth muscle cells in the pregnant uterus of the guinea pigs have shown a gradual increase in the content of collagen in the intercellular spaces The latter is relatively most pronounced in the horns (especially the pregnant one) as in the normal cervix a rather high content of collagen already is present In the pregnant uterus the collagen has a very loose arrangement whereas in the normal animal it is present in dense boundles During the course of pregnancy there is a gradual increase in the amount of the intercellular ground substance as shown by the Hale reaction the alcain blue staining and the PAS reaction This indicates that a production of mucopolysaccharides especially acid mucopolysaccharides has taken place Although the positivety in the various histochemical reactions are not very striking and does not indicate a high concentration of these substances quantitatively it may be a very pronounced increase of the mucopolysaccharides of the ground substance owing to the size and greater extent of the intercellular spaces in comparison with the normal control animals Further more quantitative and qualitative changes in the uterine mast cells were observed The number of mast cells gradually increases towards the end of the pregnancy especially in the cervix and at the same time degranulation and metachromatic halo are seen

The hypertrophy of the individual muscle cells seen by light- and electron-microscopy is in accordance with the findings of *Krichesky* (14) and *Maibenco* (15) Both of these authors found by ordinary light microscopy a hypertrophy and no hyperplasia of the musculature during pregnancy contrary to *Stieve* (25) who also saw proliferation of muscle cells However, although we did not see any mitoses without colchicin treatment, we cannot rule out that proliferation of muscle cells indeed occurs

Maibenco (15) found an increase of the intercellular substance in the uteri of pregnant rats, especially at term and mostly chemically related to neutral mucopolysaccharides Our investigations in guinea pigs seem to indicate, that there is an increase also of acid mucopolysaccharides during pregnancy The enzymatic degradation of the positive colour reactions by hyaluronidases speaks in the favour of such an assumption

The increase in the total amount of collagen corresponding to the age of the pregnancy is in accordance with the biochemical determinations made by *Harkness* and co-workers (6, 7, 8) When the hydroxyproline values were taken as a measure for the amount of collagen, a definite increase in comparison with the normal values was seen as regards collagen in the pregnant horn and a lesser increase in the non-pregnant horn and in the cervix

Our electron-microscopic investigations confirm these findings, but show furthermore that a change in the structure of collagen takes place As mentioned above the collagen in the pregnant animals has a diffuse, loose arrangement and a widespread distribution This is in accordance with the observations of *Hughesdon* (9) He described the fibro-muscular structure in the human pregnant uterus in ordinary histologic preparations and found that the collagen fibrils became separated by oedema Contrary to him we did not find any signs of resorption and subsequent decrease in the total amount of collagen as long as the pregnancy is maintained

Harkness & Harkness (6) were of the opinion that the increase of collagen in the pregnant uterus might be due to streching of the uterine wall owing to the growth of the foetus They found this statement confirmed by the fact that the collagen content predominantly is increased in the pregnant horn However, these authors thought that an additional hormonal factor was co-operating, as the non-pregnant horn in the first ten days of pregnancy shows levels of collagen content much above those from normal animals

According to *Reynolds* (23) a constant relationship exists between the condition of the uterus and the size of its content This relationship possibly was governed by "tissue tension, muscle thickness and hormonal stimuli"

It seems as if the hormonal stimuli on the uterus are governed by the balance between the sex hormones, especially ostrogen/progesterone, at

a given time Towards the end of pregnancy there is an increase of this ratio, mainly due to increase of oestrogen

Iversen in a previous publication (11) demonstrated, that the amount of collagen in uteri of guinea pigs increases during treatment with oestrogens He also showed that in human cervix uteri an increase of the ratio hyaluronic acid:chondroitin sulfate is found during oestrogen treatment (10) It therefore is assumed, that hormonal stimuli is of greater importance than thought by previous investigators of the problem The changes in the uterine connective tissue during pregnancy, *i e* increase of collagen and alterations of its structure, to some extent should be dependent on hormonal influences

The changes of the mast cell occurence and morphology at the end of pregnancy correspond to similar changes described by *Iversen* (11) for uteri of guinea pigs after oestrogen treatment of short duration Although it is not our intention to discuss the problems about mast cell morphology and function, we feel justified to emphasize that the observed morphological changes run parallel to an increase of the ground substance (compare *Asboe-Hansen* (1)), so that it is possible that at least a part of this substance arises from these cells It is striking that the increase of the number of mast cells and their degranulation followed by the appearance of an extracellular metachromatic halo predominantly is seen in the uterus at term And it is known that the collagen very early, within 6 to 12 hours after delivery, begins to be resorbed Possibly the mast cells in some way facilitate this resorption of collagen

The pyroninophilia of the mast cell cytoplasm seemed to be due to ribonucleic acid, as it was removable with ribonuclease This finding corresponds to the presence of ribosomes in the cells of a mouse mastocytoma studied by electron-microscopy (12)

SUMMARY

The myometrium in pregnant guinea pigs, especially of the cervix, were investigated by histological and histochemical methods and by electron-microscopy An increase of collagen in the interstitial spaces gradually takes place This must partly be due to an increase in the widths of the intercellular spaces, as the collagen at the same time becomes structurally altered The collagen in the pregnant animals was loosely arranged apparently lying in a more ample ground substance The interstitial cells showed marked hypertrophic changes, which should be related to production of fibrils and mucoproteins and mucopolysaccharides, particularly acid mucopolysaccharides The changes seen in the arrangement of collagen was especially distinct by electron-microscopy The number of mast cells increased towards the end of pregnancy, and morphologically these cells showed degranulation and were surrounded by a metachromatic halo

REFERENCES

1 *Asboe Hansen G* Endocrine Control of Connective Tissue Amer J Med 26 470 1959

2 *Bloom, G & Kelly J W* The Copper Phthalocyanin Dye "Astrablau and its staining Properties especially the Staining of Mast Cells Histochemie 2 48–57, 1960

3 *Christensen H F Iversen O H & Rask-Nielsen R* Studies on a transplantable Mastocytoma in Mice II Electron microscopic observations To be published, J nat Cancer inst

4 *Christensen H F Hjorth G H & Iversen O H* Studies on the Cytochemistry and Ultrastructure of the Foa Kurloff cells in the guinea pig Reports from the third Scandinavian Conference on Cell Research Copenhagen 1962

5 *Eslelund V* Fast Two step Trichrome Staining for Paraffin and Frozen Sections Combining Demonstration of Elastic Fibres, Fibrin and Mucus with v Gieson Stain Acta path et microbiol scandinav 40 105 1957

6 *Harkness M L R & Harkness R D* The Collagen Content of the reproductive Tract of the Rat during Pregnancy and Lactation J Physiol 123 492 500 1954

7 *Harkness, M L R & Harkness R D* The Distribution of the Growth of Collagen in the Uterus of the pregnant Rat J Physiol 132 492 501 1956

8 *Harkness M L R & Harkness R D* Changes in the physical Properties of the uterine Cervix of the Rat during Pregnancy J Physiol 148 524 547 1959

9 *Hughesdon P F* The fibromuscular Structure of the Cervix and its Changes during Pregnancy and Labour J obst and gynæc 59 763–776 1952

10 *Iversen O H & Marcher K* The Degree of Polymerisation of Hyaluronic Acid isolated from the Myometrium of the human Cervix Uteri Acta Pharm et Toxicol 18 101–104 1961

11 *Iversen O H* The Influence of estrogenic and androgenic Hormones on Mast cells and Connective Tissue in the Uterus of Guinea Pig Acta path et microbiol scandinav 56 245 250 1963

12 *Iversen O H & Christensen H F* Electron Microscopic Appearance of the Myometrium of Cervix Uteri in Castrated Guinea Pigs treated with sex Hormones To be published Acta path et microbiol scandinav

13 *Keiffer H* The anatomical Condition of the Wall of the Uterus of lesser Mammals at term J Obst Gynaec Brit Emp 30 331 1923

14 *Krichesky B* A histologic Analysis of the Uterine Growth during Pregnancy in the Rabbit Anat Rec 82 551–564 1942

15 *Maibenco H G* Connective Tissue Changes in postpartum Uterine Involution in the albino Rat Anat Rec 136 59 72 1960

16 *Marshall* An Outline of the Cytology and Pathology of the Reticular Tissue Oliver & Boyd London 1956 p 258

17 *Montfort I & Perez Tamayo R* Studies on Uterine Collagen during Pregnancy and Puerperium Lab Invest 10 1240–1258 1961

18 *Mowry R W* Improved Procedure for the Staining of Acid Polysaccharides by Mullers Colloidal (Hydrous)Ferric Oxide and its Combination with the Feulgen and the Periodic Acid Schiff Reaction Lab Invest 7 566 1958

19 *Newman Borysko & Swerdlow* J Res Natl Bur Standards 43 183 1949

20 *Palade* Study of Fixation for Electron Microscopy J Exp med 95 285 298 1952

21 *Pearse F* Histochemistry J & A Churchill Ltd London 1960 pp 831 825

22 *Pease Daniel C* Histological Techniques for Electron Microscopy Academical Press New York and London 1960 pp 141 143

23 *Reynolds S R M* Gestation Mechanisms Ann New York Acad Science 75 691 699 1959

24 *Spicer S S* Siderosis associated with increased Lipofucsins and Mast cells in aging Mice The amer J Path 37 457 462 1960

25 *Stieve H* Über die Neubildung von Muskelzellen in der Wand der schwangeren ... fur Gynakologie 56 1442 51 1932

26 ... A superior imbetting Medium for ... uman Epidermis The Journal of

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UNIVERSITETETS INSTITUTT FOR TROMBOSEFORSKNING RIKSHOSPITALET OSLO HEAD PAUL A OWREN MD

THE PLATELET PLUG IN NORMAL PERSONS

2 *The Histological Appearance of the Plug in the Secondary Bleeding Time Test*

By

LEIF JORGENSEN and CHRISTIAN F BORCHGREVINK

Received 8 xi 62

In 1958 *Borchgrevink & Waaler* introduced the determination of the *secondary bleeding time*, originally devised as a screening test for haemophilia While patients with defective intrinsic clotting system usually had normal bleeding time by conventional tests they bled definitely longer if the crust of the test wound was gently removed 24 hours later Normal persons and patients with defective extrinsic clotting system bled even shorter the second time than the first From this it was concluded that the intrinsic clotting system is of great importance to the secondary haemostasis in contrast to the minor role it plays in the primary haemostasis

In subsequent articles (*Borchgrevink* 1961 a *Borchgrevink & Owren* 1961 *Owren & Borchgrevink* 1962) it was claimed that the normal haemostasis during the secondary bleeding depends on a normal intrinsic clotting system on the platelet surface The tissue thromboplastin which is released by the original cut and which triggers the extrinsic clotting system will have become inactivated 24 hours later No more tissue thromboplastin is supposed to be released by the provocation of the renewed bleeding

Borchgrevink (1961 a) also presented several other observations which support the theory of a difference between the primary and secondary haemostasis in normal persons

The platelet count in the first drop of blood during the primary bleeding was 30 50 per cent lower than in venous blood and it remained fairly stable from drop to drop until just before the bleeding stopped In the secondary bleeding the platelet count in the first drop was about the same as in the venous blood and then it decreased steadily from drop to drop until the bleeding stopped

In the primary bleeding the first drop formed in about 40 seconds and the maximum blood loss occurred only after 2 minutes In the

secondary bleeding the first drop formed in 5–10 seconds and the maximum blood loss was found initially

The patterns of both platelet consumption and blood loss during the primary bleeding became similar to those of the secondary bleeding if the vessels were dilated by warming the skin prior to the test Consequently, the dissimilarity of the patterns in the two types of bleeding may be due to differences in vascular tonus

Finally, *Borchgrevink* (1961 a) found a marked difference in the total number of platelets retained in the wound during the two types of bleeding In the primary bleeding 11 million platelets, on an average, were supposed to be consumed in the formation of platelet plugs, whereas in the secondary bleeding the corresponding figure was only 3 millions

In a previous article (*Jorgensen & Borchgrevink* 1963 a) the histological picture of the normal platelet plug was described, as seen in wounds of the *primary* bleeding time test Fifteen to twenty minutes after the bleeding the plugs were composed of ballooned discrete platelets and bordered by a perimetric fibrin membrane The gap of the wounds was filled by erythrocytes, partly trapped in a delicate fibrin net No platelets were intermingled with the red blood cells apart from tiny particles of platelet origin attached to this net Twenty-four hours after the bleeding there was a considerable increase in the amount of fibrin, both in the plugs and in the gap of the wounds In the surrounding tissue an acute inflammatory reaction was evident

In this article we intend to examine histologically the wound of the secondary bleeding time test in normal persons 15–20 minutes after the bleeding We want to see if the physiological differences between the primary and secondary haemostasis are reflected by morphological distinctions

A subsequent paper (*Jorgensen & Borchgrevink* 1963 b) will deal with the histological features of both the primary and secondary haemostasis in patients with haemorrhagic diseases, including coagulation defects

MATERIALS AND METHODS

Determination of the primary and secondary bleeding time was performed in four normal persons All were men aged 24 to 36 years (Table 1) In all the bleeding times as well as the platelet count was within the normal range

The method used for the determination of the *primary bleeding time* is a modification of the technique described by *Ivy Nelson & Bucher* (1941) developed by *Borchgrevink & Waaler* (1958) A blood pressure cuff is placed on the upper arm and inflated to 40 mm Hg On the volar side of the forearm a transverse cut 1 mm deep and 10 mm long is made with a new surgical blade Every 30 second the shed blood is carefully absorbed by a filter paper until the bleeding stops The normal range for the primary bleeding time with this method is between 3 and 11 minutes

The *secondary bleeding time* was determined by provoking a renewed bleeding in the same cut 24 hours later (*Borchgrevink & Waaler* 1958) The blood pressure cuff is reapplied and inflated as before The crust of the wound is gently removed with a surgical blade great care being taken neither to cut new vessels nor to cause new tissue damage The time until the bleeding stops is recorded in the same manner

as before Normal values for the secondary bleeding time are between 0 and 6 minutes

Biopsies Fifteen to twenty minutes after the cessation of the secondary bleeding the wound together with the surrounding skin was excised under local anaesthesia with xylocain 1 per cent solution

Histological technique The biopsy specimens were immediately transferred to Helly s solution for fixation for 24 hours Afterwards the specimens were rinsed in flowing tap water for another 24 hours imbedded in paraffin, and cut in serial sections about 5 microns thick perpendicular to the direction of the wound Following stains were used

(1) Haematoxylin and a solution of eosin Y (1 per cent), orange G (0 25 per cent), and acid fuchsin (0 125 per cent) in 96 per cent ethyl alcohol and 0 05 per cent acetic acid The platelets stained pale red the fibrin deep red

(2) Lendrum s acid picro Mallory method for staining fibrin (*Lendrum* 1949) The platelets stained light blue or bluish red the fibrin red or slightly yellowish red

(3) Mallory s phosphotungstic acid haematoxylin method The platelets stained light brown the fibrin dark blue

TABLE 1

Age Primary and Secondary Bleeding Time and Platelet Count in the 4 Normal Men from whom the Biopsies Were Taken

Person	Age in years	Primary bleeding time in minutes	Secondary bleeding time in minutes	Platelet count per cu mm blood
M H	31	6	4	271 000
D H	29	5	2½	248 000
H S	24	5½	3	264 000
C B	36	7	3½	237 000

RESULTS

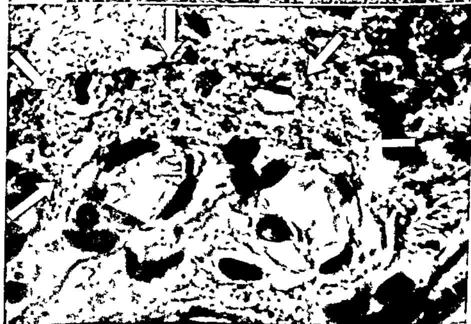
There was no important difference between the four wounds, allowing a joint histological account The features which remained of the primary haemostasis have already been described (*Jorgensen & Borchgrevink* 1963 a) The crust and the upper one or two thirds of the fibrin mass in the gap of the wound were removed (Fig 1) Renewed bleeding had occurred from capillaries and smaller capillary veins, definite arteriolar bleeding was not found Many of the vessels which had bled were dilated, but some of them were rather collapsed

The denuded vascular mouths, even those of the capillaries were

... ly plugs the form varied considerably, but the size was definitely smaller compared with primary plugs belonging to vessels with the same diameter of the lumen The largest secondary plugs had a diameter about 3 4 times that of the lumen

Many of the plugs were composed of aggregated granular platelets with a diameter of 1 2 microns Usually the platelets seemed to have fused (Fig 2), but in the centre of some of the larger plugs the plate-

1 

2 

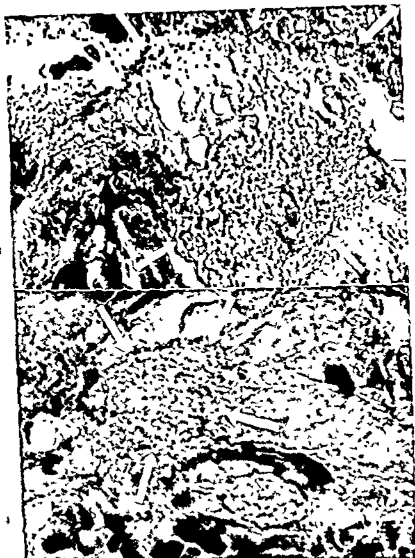
Figs 1 and 2

Fig 1 The secondary wound The skin surface to the left The superficial crust and some of the old fibrin net in the gap of the wound are removed The rest of the old net is in the bottom of the wound to the right and along the upper wound surface in the middle Between the arrows at upper left is a large secondary plug belonging to a dilated capillary or capillary vein Lendrum's stain × 140

Fig 2 One flattened granular indistinctly outlined plug covers the mouths of two dilated capillaries The platelets have apparently fused A few red and white blood cells are included in the plug No perimetric fibrin membrane Lendrum's stain × 800

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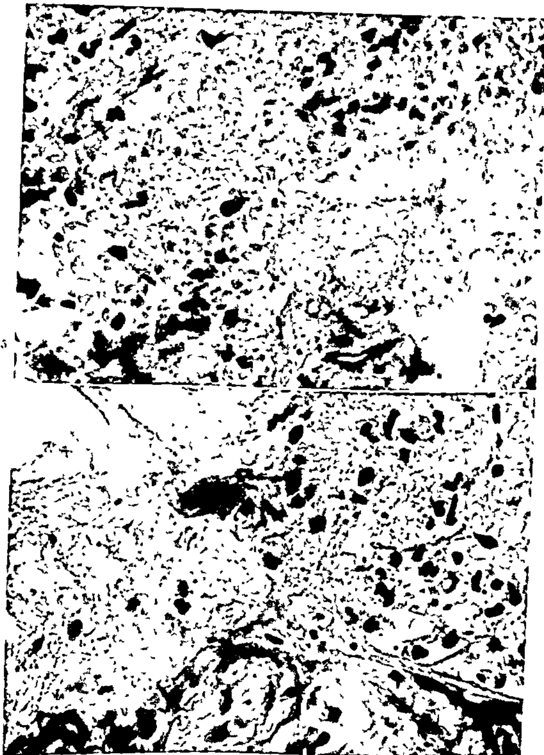
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Figs 3 and 4

Fig 3 The large plug seen in Fig 1 The wound surface to the left Within the plug there is an area of more scattered platelets with distinct granulomers The clear distinctly bordered spaces are artifacts Towards the periphery particularly to the lower left the platelets are more densely *aggregated and* apparently fused At the three upper arrows a hazy perimetric zone stains as fibrin Lendrum's stain × 800

Fig 4 An indistinctly outlined plug belonging to a dilated capillary at the bottom. Some of t[illegible]
assume t[illegible]
is develop[illegible]
at upper [illegible]



Figs 5 and 6

Fig 5 A saucer-shaped plug with ballooned platelets and distinct perimetric fibrin membrane, fastened to the wound surface at the bottom of the picture The corresponding vessel is not clearly shown Many platelets are intermingled with the extravasated erythrocytes Lendrum s stain × 400

Fig 6 To the left a rich, newly formed fibrin net to the right old fibrin strands In the masks of the old net are aggregates of apparently fused platelets, as well as red and white blood cells Lendrum s stain × 400

lets, containing a distinct granulomer, were more scattered, separated by structureless, faintly eosinophil material, appearing as plasma (Fig 3) Other plugs, not so often observed, had a structure like the primary plugs, although the ballooned platelets were not very large (Fig 5) Finally, some of the plugs were in various stages of transition between the granular and multivesicular forms (Fig 4)

The outlines of the granular plugs were often indistinct due to blending with platelet aggregates in the haematoma of the gap The fibrin membrane was mostly lacking, apart from an occasional incomplete, hazy peripheral band (Fig 3) In the multivesicular plugs a distinct perimetric fibrin membrane was present (Fig 5), in the transitional plugs it was only faintly indicated (Fig 4)

A few leucocytes or erythrocytes were observed at times within the plugs, but definite remnants of channels were not encountered

The outer part of the *gap of the wound*, which had been exposed at the provocation of the secondary bleeding, was partly refilled by erythrocytes entrapped in a rich fibrin net (Fig 6) This was much more conspicuous and constant than that in the haematoma of the primary bleeding at the same time after the test Numerous platelets were attached to it and, in addition, individual or aggregated platelets were often intimately mixed with the red blood cells, in contrast to the sparsity of platelets in the primary haematoma Partly fused platelet aggregates were also prone to appear on denuded parts of the walls in between the vessels, and in the masks of the coarse fibrin net from the previous day in the deeper parts of the wound (Fig 6)

DISCUSSION

Many of the secondary plugs were composed of aggregated granular platelets and they were mostly lacking any fibrin This gives histological support to the widely held view, particularly maintained by *Zucker* (1947) and *Hugues* (1953), that visible fibrin formation is not necessary for the arrest of bleeding However, small amounts of thrombin on the platelet surface is considered necessary for the "viscous metamorphosis" and the impermeability of the platelet plug (*Luscher* 1956, *Borchgrevink* 1961 b, *Borchgrevink & Owren* 1961) These phenomena are usually associated with apparent fusion of the platelets, a process which, at least partially, had taken place in all secondary plugs of this type

In [illegible] 1963 a) we described
su [illegible] the superficial crust
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represent a later stage in the post-haemorrhagic transformation of platelet aggregates The aggregates of the crust were probably fresher than the multivesicular plugs Support of this interpretation is ob-

tained in this study by the observation of several secondary plugs in various stages of transformation from granular to multivesicular plugs The perimetric fibrin membrane seemed to develop along with the ballooning of the platelets, an observation previously made by *Sharp* (1961) in experiments *in vitro*

Thus, we may assume that the secondary plugs in general were fresher than the primary ones, *i e* completed closer to the grossly observed arrest of the bleeding This theory is supported by two additional facts Traces of traversing channels through the secondary plugs were not found, indicating that the plugs became impermeable just after their completion Further, the high percentage of retained platelets observed during the entire primary bleeding was reached only at the end of the secondary bleeding (*Borchgrevink* 1961 a)

These differences between the primary and secondary haemostasis need not to be explained by referring to the assumed lack of tissue thromboplastin in the secondary wound They may be due to the *difference in vascular tonus,* because the dissimilarity of platelet consumption is obliterated simply by warming the skin prior to the primary bleeding time test (*Borchgrevink* 1961 a) Around the secondary wound there was a vascular dilatation as a part of an acute inflammatory reaction to the cut on the previous day This explains the massive bleeding immediately after the removal of the crust In this sudden gush the platelets probably do not have the same opportunity to adhere to the vessels and to aggregate as during the slower initial flow of the primary bleeding Many of the not adhered platelets were included in the secondary haematoma, contrasting with the few platelets in the primary haematoma

Another important difference between the primary and secondary wound is that the latter was *more superficial* than the former Renewed bleeding had only occurred from capillaries and smaller capillary veins This may account for the fact that the secondary bleeding time is somewhat shorter than the primary (*Borchgrevink & Waaler* 1958), although the vascular dilatation should predispose to a prolonged bleeding time (*Roskam* 1945) However, we do not know when the primary bleeding from these superficial vessels stopped their primary bleeding time may even be shorter than the secondary It is not unlikely that during the later stages of the gross primary bleeding only the deeper and larger vessels are bleeding In favour of this supposition is the lack of channels in the superficial primary plugs, whereas the deeper and larger primary plugs had several traces of these structures (*Jorgensen & Borchgrevink* 1963 a)

When comparing the diameter of the platelet plugs with that of the vascular lumen, the secondary plugs were considerably smaller than the primary ones Admittedly, the secondary plugs were generally composed of less swollen platelets, a fact which may exaggerate the difference Further, the difference in size would perhaps be less marked

if the comparison was made between plugs belonging to the same kind of vessel However, this does probably not account for the entire difference The smaller size of the secondary plugs is in accordance with the generally lower platelet consumption throughout the secondary bleeding, and also with the considerably smaller total number of platelets retained This latter figure is certainly also influenced by the shorter secondary bleeding time

A third fundamental difference between the primary and secondary wound is revealed by the *more conspicuous fibrin network* of the latter at the same time after the bleeding This is probably the morphological counterpart of the observation of a shorter clotting time in blood from the secondary bleeding than in that from the primary (*Borchgrevink* 1963) The reason for this may only be the subject of conjecture influence of the inflammatory exudate, of the serum derived from prolonged or continuous coagulation in the primary wound, or of the increased number of platelets in the secondary haematoma

There is an apparent contrast between the rich fibrin net of the secondary wound and the platelet plugs which have generally not reached very far in their post haemorrhagic development This must mean that the coagulation within the haematoma and within the plugs is largely independent, and it supports the theory that the thrombin necessary for the impermeability of the plugs is formed on the platelet surface (*Borchgrevink* 1961 b, *Borchgrevink* & *Owren* 1961, *Owren* & *Borchgrevink* 1962)

It should be stressed that we have not found any histological feature of the secondary wound which clearly reflects the assumed lack of tissue thromboplastin However, this may indicate that an intact intrinsic clotting system is able to compensate completely for this defect

SUMMARY AND CONCLUSIONS

Determination of the secondary bleeding time was performed in four normal persons and biopsies of the wounds were taken 15 20 minutes after the bleeding The crust and the upper parts of the fibrin mass of the wound from the previous day had been removed Renewed bleeding had occurred from superficial capillaries and smaller veins, many of which were dilated as a part of an inflammatory reaction to the cut of the primary bleeding time test

Fresh platelet plugs covered the denuded vascular mouths Compared with the vascular diameter, the secondary plugs were smaller than the primary ones Many of them were composed of aggregated granular platelets, all of which had apparently fused, except within the centre of some of the larger plugs Other plugs had a multivesicular appearance or a structure representing various stages in between these two forms In the granular plugs a perimetric fibrin membrane was mostly lacking it seemed to develop along with the ballooning of the platelets

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bleeding and its role in the capillary haemostasis Acta path et microbiol scandinav 57 40–56 1963 a

Jørgensen L & *Borchgrevink C F* The haemostatic mechanism in patients with haemorrhagic diseases A histological study of wounds made for primary and secondary bleeding time tests In preparation 1963 b

Lendrum A C The staining of erythrocytes in tissue sections A new method and observations on some of the modified Mallory connective tissue stains J Path Bact *61* 443 448 1949

Lüscher E F Viscous metamorphosis of blood platelets and clot retraction Vox sanguinis *1* (N S) 133 154 1956

Owren P A & *Borchgrevink C* The hemostatic function of plasma clotting factors adsorbed on the platelets Paper no 356 in Proceedings of the Eighth Congress of the European Society of Haematology Vienna 1961 Basel & New York S Karger 1962

Roskam J Les bases pathogéniques de la thérapeutique hémostatique chez les saigneurs Arch int Pharmacodyn *71* 389 410 1945

Sharp A A Platelet (viscous) metamorphosis Pp 67 88 in *Johnson S A* *Monto* [illegible]

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[illegible] 148 [illegible] 1947

In the gap of the wounds a rich fibrin network entrapped the erythrocytes, which were mixed with many platelets

The following *conclusions* are drawn.

The occurrence of granular plugs without fibrin supports the view that visible fibrin precipitation is not necessary for the arrest of bleeding

In general, the secondary plugs are not so far in their post-haemorrhagic development as the primary plugs at the same time after the bleeding has stopped The secondary plugs are probably completed closer to the grossly observed arrest than are the primary ones

The many platelets in the haematoma and the small size of the plugs probably illustrate the low platelet consumption during the secondary bleeding

Both the relatively late completion of the plugs and the low platelet consumption may be explained by the inflammatory vascular dilatation

The smaller total number of consumed platelets is also partly explained by the shorter secondary bleeding time This may again be accounted for by the fact that only the superficial vessels bleed the second time

The conspicuous fibrin network probably reflects the shorter clotting time in the blood from the secondary bleeding

The apparent contrast between the rich fibrin network and the not far developed platelet plugs supports the theory that thrombin necessary for the impermeability of the plugs is formed on the platelet surface, independent of the coagulation in the haematoma

The assumed lack of tissue thromboplastin in the secondary wound is not normally reflected by any histological feature

REFERENCES

Borchgrevink C F Platelet adhesion in vivo during secondary bleeding in normal individuals and in patients with clotting defects Acta med scandinav *170* 245 254 1961 a

Borchgrevink C F The mechanism of the prolonged bleeding time provoked by dextran sulphate or by excessive doses of heparin Acta med scandinav *170* 365–373 1961 b

Borchgrevink C F Unpublished data 1963

[illegible] The *hemostatic effect* of normal platelets in [illegible] shed

[illegible]

1958

Hugues J Contribution à l etude des facteurs vasculaires et sanguins dans l hémostase spontanee *Arch* Internat Physiol *61* 565 711 1953

Ivy A C Nelson D & Bucher G The standardization of certain factors in the cutaneous 'venostasis' bleeding time technique J Lab clin Med *26* 1812 1822 1941

Jorgensen L & Borchgrevink C F The platelet plug in normal persons 1 The histological appearance of the plug 15 to 20 minutes and 24 hours after the

bleeding and its rôle in the capillary haemostasis Acta path et microbiol scand nav 57 40-56 1963 a

Jørgensen L. & Borchgrevink C F The haemostatic mechanism in patients with haemorrhagic diseases A histological study of wounds made for primary and secondary bleeding time tests In preparation 1963 b

Lendrum A C The staining of erythrocytes in tissue sections A new method and *observations on some of the modified Mallory connective tissue stains J Path* Bact 61 443 448 1949

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Owren P A & Borchgrevink C The hemostatic function of plasma clotting factors adsorbed on the platelets Paper no 356 in Proceedings of the Eighth Congress of the European Society of Haematology Vienna 1961 Basel & New York S Karger 1962

Roskam J Les bases pathogeniques de la therapeutique hemostatique chez les saigneurs Arch int Pharmacodyn *71* 389 410 1945

Sharp A A Platelet (viscous) metamorphosis *Pp 67 88 in Johnson S A Monto R W Rebuck J W & Horn R C Jr* eds Henry Ford Hospital International Symposium Blood Platelets London J & A Churchill Ltd 1961

Zucker M B Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis in normal thrombocytopenic heparinized and hypoprothrombinemic rats Am J Physiol *148* 275-288 1947

FROM THE UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE THE GADE INSTITUTE, DEPARTMENT OF MICROBIOLOGY, BERGEN NORWAY

IDENTIFICATION OF STAPHYLOCOCCUS AUREUS ANTIGENS AND ANTIBODIES BY MEANS OF THE GEL PRECIPITATION TECHNIQUE

By

PER OEDING and GUNNAR HAUKENES

Received 5 xi 62

The gel precipitation technique has been used to a certain extent for the purpose of studying antigenic substances present in *Staphylococcus aureus* and serum antibodies reacting with staphylococcal materials When potent whole sera and whole staphylococci, or crude extracts, diffuse towards each other, a very complex system of precipitation lines appears A few of these lines have been characterized to some degree, in that they have been correlated with a staphylococcal toxin (1, 2, 23, 28), an agglutinogen (16) and an antibody present in normal human (17) or animal sera (4) Furthermore, a line specific for polysaccharide A has been demonstrated (15, 7, 8), and lines characteristic of Cowan's strains I, II, and III have been reported (3)

The current interest in staphylococcal serology and immunology, and the frequent use of the gel precipitation technique in this research, make it desirable to characterize the lines which have been identified, in such a way that they may be produced and recognized by any worker who has the standard system and a description of the standard conditions The purpose of the present report is to collect and discuss our own observations some of which have already been reported In addition some lines described by other authors have been examined by us and are discussed here

METHODS

Strains The strains of *Staph aureus* used in the majority of the experiments reported here belong to the set of 13 type strains used in this laboratory for serological typing of staphylococci by slide agglutination (16) In addition strains from our routine material were used in certain experiments

Rabbit immune sera were produced by the intravenous injection of formalin killed organisms and *factor sera* by absorptions as described by *Oeding* (22) When occasionally, more concentrated factor sera were needed than the 1 in 10 standard

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dilution the factor sera were concentrated by evaporation *in vacuo* or undiluted whole sera were absorbed

Agar precipitation A modification of Ouchterlony's method with 1 5 per cent water agar containing 0 85 per cent NaCl and buffered to pH 7 4 was used (16) It is important that the antisera have a good titre of the antibody in question Whole bacteria crude extracts obtained from bacteria crushed in a bacteria press and more or less purified materials were used as antigens (see below) The bacteria were grown on nutrient agar plates

Ring test precipitation was performed with dilutions of the antigen in saline layered over undiluted immune serum (15)

Agglutination was carried out on slides with live bacteria as described by Oeding (22)

EXPERIMENTAL

Of the 9 "specific" antigenic factors used by us for the typing of *Staph aureus* strains by slide agglutination, 3 form specific and characteristic lines on agar precipitation We have been unable to demonstrate lines corresponding to the remaining 6 antigens, either with whole rabbit immune sera, or with concentrated factor sera One of the 3 lines (*n*) has been described before (16) whereas the other 2 have not

n Line

The *n* line was first described by *Haukenes & Oeding* (16) and corresponds to the *n* antigen found on agglutination

Reference system Suspensions of whole or crushed 1503 bacteria give a distinct line with 1503 rabbit immune serum, preferably undiluted The line can also be produced with *n* factor serum, but the whole serum should not be diluted more than 1 in 5 As the line is strongest with whole serum, this is preferred in the reference system As a control, serum 3189 should be placed in the well next to serum 1503, because the *n* line is the only line not common to these two systems Although the *n* line may be produced with any strain possessing the *n* antigen, the reference system is to be preferred

The serological pattern of strain 1503 is *aemn* and the phage pattern *52 52A 80 81 82 7 42E/54 73/81* *n* factor serum is produced by the absorption of Cowan III serum with 2095 and F21 bacteria

The *n* antigen is heat labile and destroyed by trypsin and is probably a protein

The *n* line is very fine and well defined, appears after 2 3 days' incubation, lies near the antigen well, and is slightly concave towards it (Fig 1) After autoclaving and trypsin digestion of the organisms no *n* line is found

Among our type strains 1503, 2253, 28, 3647 and Cowan III give an *n* line This is in accordance with the results of agglutination Strain S 365 has been shown by absorption to have small amounts of *n* antigen, but this strain gives neither agglutination in *n* serum nor the *n* precipitation line

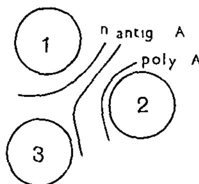


Fig 1

Schematic drawing showing the position of the *n* antigen A (Jensen), and polysaccharide A lines The latter line will usually have disappeared into the serum well when the former lines are fully developed In well 1 crushed 1503 cells in well 2 serum 1503 and in well 3 *n* factor serum

h Line

This line was identified with the *h* agglutinogen some years ago, but the observation has not been reported before.

Reference system 17A whole bacteria or a suspension of crushed bacteria will give a distinct *h* line with most 17A rabbit immune sera, preferably undiluted The line can also be produced with *h* factor serum, but the whole serum should not be diluted more than 1 in 2 As the line is weaker with factor serum, this should not be used in the reference system

The serological pattern of strain 17A is *ah* and the phage pattern *29/52/52A/80/81* *h* factor serum is produced by absorption of 17A serum with 1503 and 3647 bacteria

The *h* line appears after 2–3 days as a relatively well defined line near the antigen well, towards which it may be slightly concave When factor serum is used, the line is found near the serum basin as the antibody is weak (Fig 2)

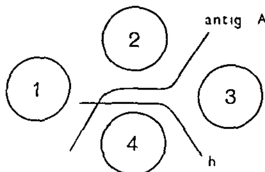


Fig 2

Schematic drawing of the *h* and antigen A (Jensen) lines in absorbed and unabsorbed sera In well 1 serum 1503 in well 2 serum 17A in well 3 serum 17A absorbed with 1503 and 3647 bacteria and in well 4 crushed 17A cells

According to the results of agglutination strain 17A is the only one of our type strains which has the *h* antigen The *h* line is found only in this strain Of other strains tested, those which agglutinated in the *h* serum all produced the line, whereas none of the non-agglutinating strains did so

Haukenes (unpublished data) has recently demonstrated by agglutinin absorption experiments that the *h* factor serum consists of two separate antibodies The corresponding antigenic factors have been designated h_1 and h_2 The former is heat labile whereas the latter is heat stable Of the type strains, 17A has both h_1 and h_2, whereas Cowan I and Cowan II have been shown to have only a weak h_2 The h_2 antigen in these strains was revealed when the agglutination was carried out with mannitol-salt agar cultures These data, and the results of agar precipitation with other h_1 and h_2 strains, show that the h_1 antigen is the one responsible for the line It is interesting that fluorescent antibody staining also distinguishes the h_1 antigen from h_2 and thus confirms the results obtained from agglutinin absorption and agar precipitation (5)

i Line

This line was identified with the *i* agglutinogen by us some years ago A short description of the line has been given by *Haukenes* (8)

Reference system Suspensions of whole or crushed F21 bacteria give a distinct *i* line with F21 rabbit immune serum, preferably undiluted The line can also be produced with *i* factor serum, undiluted in relation to whole serum, but the line is then weak and not so easily demonstrated

The serological pattern of strain F21 is *bcim* and the phage pattern *3A/3B 3C* *i* factor serum is produced by absorption of F21 serum with 1503 and 3647 bacteria The *i* antigen is heat labile and probably has a protein structure

The *i* line appears after 2 4 days, is somewhat diffuse, and situated near the antigen well towards which it is slightly concave

The results of agglutination show that only type strains F21 and Wood 46 have the *i* antigen The *i* line is found only in these strains Of other strains tested those which agglutinated in the *i* serum all produced the line whereas none of the non-agglutinating strains did According to agglutinin absorption experiments performed by *Haukenes* (unpublished data) the *i* factor serum consists of two separate antibodies The corresponding antigenic factors have been designated i_1 and i_2 Of the type strains F21 has both factors whereas Wood 46 has i_1 only It is therefore evident that i_1 is the factor responsible for the line As we have not yet been able to produce an i_2 factor serum of satisfactory titre other strains with i_1 and i_2 factors have not been tested

Polysaccharide A Line

Two "group" antigens, polysaccharide A and antigen A (Jensen), produce characteristic and easily recognizable lines which will be described here. In addition we have regularly observed two or three lines which probably represent other group antigens. These lines have, however, not yet been identified.

The polysaccharide A line has been described briefly by *Oeding* (24) and *Haukenes et al.* (15) and in detail by *Haukenes* (7, 8). It has been identified as the group-specific carbohydrate originally described by *Julianelle & Wieghard* (21) and now purified and characterized chemically and serologically by *Haukenes* (7–13).

Reference system. Wood 46 whole bacteria, extracts of crushed or whole bacteria, and purified polysaccharide A material will all give a distinct polysaccharide A line with Wood 46 undiluted immune serum. Strain Wood 46 is well suited as reference because it is very rich in polysaccharide A, lacks antigen A (Jensen), and has only *i* of the "type specific" antigens. A factor serum against polysaccharide A has not yet been produced, because the purified substance is non-antigenic, and strains suitable for the absorption of whole serum have not been found.

The serological pattern of strain Wood 46 is *i*, and the phage pattern *42D w*.

The polysaccharide A line is very characteristic. It appears after one or two days very near the serum well towards which it is strongly concave. Later the line becomes less distinct and disappears into the serum well (Fig. 1). Readings should therefore be made daily during the first days. The line is best demonstrated when the serum well is filled before the antigen well. Also when a potent serum is made to diffuse towards a diluted antigen, the line does not migrate into the serum well.

Polysaccharide A has been purified by column chromatography by *Haukenes* and analyzed in detail (15, 7–13). It has been shown to be an N-acetylglucosaminyl-ribitol phosphate polymer linked to a smaller polypeptide moiety. The reactivity on ring test precipitation with undiluted whole serum was $1:4 \times 10^6$. This specific line was of great value in addition to ring test precipitation in following the purification of polysaccharide A.

It was shown (14) that teichoic acid from the walls of *Staph. aureus* strain H and polysaccharide A have identical serological reactivity. When examined by ring test precipitation against a potent *Staph. aureus* immune serum both preparations reacted to the same titre, and on agar precipitation they gave bands of identical strength and appearance showing a "reaction of identity". Although the structure of the two substances is very similar, there are interesting differences by means of which it can be shown that certain groups are not essential for the serological reactivity. Investigations are in progress to determine the serologically active grouping. So far it seems to be the β linked N-acetyl-

glucosaminyl-ribitol residues in the ribitol-phosphate polymer which determine the specificity

Polysaccharide A was found in all of our 13 type strains and has further been demonstrated in each of 100 routine *Staph aureus* strains examined In three of these strains the amount of polysaccharide A was small and could be demonstrated only by absorption procedures Thus polysaccharide A is a characteristic wall antigen in *Staph aureus* strains However, this antigen has also been demonstrated in small amounts with another group carbohydrate in some strains of *Staph epidermidis* Investigations are in progress on the polysaccharide antigens of *Staph epidermidis* (*Losnegard & Oeding*, in press)

Polysaccharide A is probably a weak agglutinogen and is not the cause of the strong cross agglutinations between strains of *Staph aureus* in non-absorbed rabbit immune sera (12)

Antigen A (Jensen) Line

This line was first described by *Jensen* (17, 18), and has later been discussed in several papers (24, 3, 4, 19, 20)

Reference system Cowan I whole bacteria, extracts of crushed or whole bacteria, and more or less purified antigen A material will all give a distinct antigen A line with undiluted normal human serum Strain Cowan I is recommended because of its high content of antigen A Cowan I rabbit immune serum also gives lines with other antigens present in Cowan I bacteria or extracts Antigen A has not been produced in a pure state and to get the antigen A line only, normal human serum should be used A factor serum against antigen A has not been produced

The serological pattern of strain Cowan I is *am* and the phage pattern 52 52A/80

The antigen A (Jensen) line appears after one or two days as a well defined and strong line situated midway between the serum and the antigen wells Its position in relation to the polysaccharide A line and the *n* line in the 1503 system is illustrated in Fig 1

Our investigations have shown that in the Cowan I system two lines develop regularly in the area of the antigen A line The line on the antigen well side gives a "reaction of identity" with the antigen A line obtained with normal human serum The line on the serum well side represents another antigen present in the partly purified antigen A extract from strain Cowan I This antigen was also found in some of the other type strains and its distribution correlated completely with one of our *a* factors (a_2) but absorption experiments did not confirm the identity *Jensen et al* (20) demonstrated the presence in extract *A* of two antigens in addition to antigen A The line on the serum well side demonstrated by us in extract A prepared according to *Jensen* (17, 18) is apparently not identical to either of the two new antigens of *Jensen et al* and seems to be a fourth antigen

Antigen A was reported by *Jensen* (17, 18) to be present in 73 out of 97 *Staph aureus* strains when examined by agglutination, whereas only 23 of the strains gave an antigen A line This indicated that a strain had to be relatively rich in antigen A to give the precipitation line Our investigations have, however, shown that all the 13 type strains, except Wood 46, and nearly all the routine strains of *Staph aureus* examined, gave an antigen A line Antigen A is thus a group antigen present in nearly all strains of *Staph aureus*, but the demonstration of the line depends upon the technique, *e g* the use of absorption when the amount of the antigen is small Antigen A has not been found in strains of *Staph epidermidis* (17, 18)

Jensen (17, 18) reported that antibodies to antigen A were present in all normal human sera, but only in some of the normal rabbit sera examined This has been confirmed by *Cohen et al* (3, 4) and by us (unpublished data) *Jensen* also found that the agglutinating property of normal human sera was due to the same antibody that produced the antigen A line *Cohen et al* (personal communication) made the interesting observation that the antibodies to antigen A were present in 8-month-old, but not in 2-month-old germ free mice The authors also examined the agglutinating capacity of various animal sera and found evidence that antigen A is not an agglutinogen If this is correct, it is not possible to check for antigen A activity by agglutination tests

According to *Jensen* (17, 18) antigen A is probably a polysaccharide present on the surface of *Staph aureus* It has a toxic effect on isolated ileum from normal guinea pigs

Examining a large number of sera from healthy persons, *Jensen* (18, 19) demonstrated other lines in addition to the antigen A line in some of them Altogether 8 lines, including the antigen A line could be distinguished using different strains of *Staph aureus* It can not be decided whether any of these lines correspond to lines described by us (polysaccharide A line, *n* line, *h* line, *i* line) It is obviously of great interest to know more about the antibodies against *Staph aureus* occurring in sera from healthy persons and patients, and the gel precipitation technique seems to be well suited for such investigations The lines observed should be compared to known lines, using the appropriate reference systems

Lines in Cowan Types I, II and III

Of the antigens described above Cowan type strains I, II and III all have polysaccharide A and antigen A (Jensen) and give the corresponding lines Strain Cowan III gives the *n* band in addition

Cohen et al (3) described what appeared to be a type specific line in each of the three Cowan type strains They were all strong lines which appeared after 18 to 30 hours We have tried to reproduce these lines in order to compare them with those described by us In our ex-

periments whole bacteria or saline suspensions of crushed bacteria were put up against the corresponding whole sera

In the Cowan I system *Cohen et al* described a line, close to the serum well which was not found in the Cowan II or III strains The position of this line indicates that it is identical to the polysaccharide A line *Cohen et al* did not observe the line in the Cowan II or Cowan III strains This may be due to the rapid migration of polysaccharide A in the agar with displacement of the line into the serum well If the peculiarities of the polysaccharide A line are not known, it may easily be overlooked, and the line, when observed in some strains, may give the impression of representing a specific antigen

In the Cowan II system *Cohen et al* described a line which they designated antigen IV This line was strong, situated between the antigen A (Jensen) line and the serum well, and was not produced by strains Cowan I or III, but by a number of other strains The antigen responsible for this line was found to be heat stable and was not precipitated at pH 3 These characteristics are similar to those of polysaccharide A The line observed in the Cowan II system may be identical to that found in the Cowan I system as different contents of antibody in the two sera and of antigen in the cells may alter the conditions for detecting the line

In recent experiments we have not been able to detect lines other than the polysaccharide A line which might correspond to the antigen IV line However in earlier experiments a strong line resembling the antigen IV line was observed in Cowan II bacteria but not in Cowan I, Cowan III or other type strains This line corresponded very well to the major agglutinogen of strain Cowan II, Cowan factor 3 (*Haukenes*, unpublished data) This factor seems, however to be relatively infrequent, whereas antigen IV appears to be common The question whether the antigen IV of *Cohen et al* is identical to the polysaccharide A line or due to an antigen characteristic of Cowan type II *e g* Cowan factor 3 must await further investigations

In the Cowan III system *Cohen et al* have described a double precipitation line close to the serum well which was not observed in the Cowan I or Cowan II strains This line cannot be the *n* line which is found near the antigen well We have not observed any line in the Cowan III system which could be the double line of *Cohen et al*

Our difficulty in reproducing and recognizing the lines described by *Cohen et al* is probably due to differences in antigens, antisera and technique *e g* in the concentration of agar which was only 0 8 per cent in Cohen et al s experiments in comparison to 1 5 per cent in ours However it should be noted that all of the three lines described by these authors are situated near the serum well and may escape detection

Lines in Normal Rabbit Serum

Cohen et al (3, 4) described a characteristic precipitation line occurring when extracts of crushed Cowan type I and III cells but not of Cowan II cells, diffused against sera from normal rabbits. This line occurred about midway between the serum and antigen wells, was thin, and weaker than most lines observed with immune serum. The antibody was demonstrated in the sera of 36 non-immunized specific-pathogen-free rabbits examined. In non-immunized rabbits from a commercial source the line was demonstrated, together with other lines. The line was not identical with the antigen A line, which does not normally occur in rabbits. Cowan type I bacteria were agglutinated by normal rabbit sera in contrast to Cowan type II and III bacteria. Absorption experiments showed that the precipitating and agglutinating antibodies were different.

Our attempts to demonstrate this normal rabbit serum line have not been successful. In one series of experiments a line, similar to that described by *Cohen et al*, was observed in three normal and one immunized rabbit against an extract of Cowan I bacteria. However, the line could not be reproduced in later experiments, even with the sera which had previously shown the line. The failure to produce the normal rabbit line may be due to different colonies of rabbits and to the method used for the production of bacterial extracts. According to *Cohen* (personal communication) this antibody has also been demonstrated in normal mice, hamsters, guinea pigs, and humans. The antigen has been shown to be truly staphylococcal.

Reference system. Normal rabbit serum, preferably from pathogen-free animals, is diffused against an extract of Cowan I or Cowan III cells prepared in the following way (*Cohen*, personal communication). The cells are grown in heart infusion broth (Difco) and washed with distilled water. One $\times 10^{11}$ or 1×10^{12} cells suspended in phosphate buffered saline at pH 7.2 are crushed in the cold. The antigen is present in the supernate after centrifugation at 10 000 $\times$ g for 1 hr, is missing from more dilute disintegrates, and is difficult to prepare.

Toxin Lines

When extracts of *Staph aureus* strains are examined by the gel precipitation technique, not only bacterial antigens, but also toxins, may produce lines. On the other hand, when working with a toxin, the appearance of lines due to bacterial antigens must be taken into consideration. Particular interest has been paid to the localization of lines corresponding to the enterotoxin (26, 1), to leucocidin (28), and to the α-toxin (6, 27, 25, 2, 23). As lines occurring in the α-toxin-antitoxin system are likely to be found in precipitation systems consisting of whole bacteria or extracts and antibacterial sera, some experience of the α-toxin system will be reported here.

When growing staphylococci (6) or Wood 46 toxins (27, 25) diffuse against commercial staphylococcus α-antitoxin, multiple precipitation lines appear *Stern & Elek* (25) found that one strong line, which was not present in a system of non-haemolytic endoplasm and antitoxin, was probably the α haemolysin line Our investigations (23) indicated that among the precipitation lines of the Wood 46 toxin-antitoxin system, two complex bands consisting of at least five distinct components were due to toxins The corresponding antibodies were not demonstrated in antibacterial rabbit immune sera The band closest to the toxin well was found to be associated with α-haemolysis This band consisted of two closely situated lines, of which the weakest, which was next to the antitoxin well, was found by ammonium sulphate fractionation to correspond to the α toxin Similar results were reached simultaneously by *Butler* (2) on ethanol fractionation of staphylococcal α-toxin

Commercial α antitoxin was shown (23) to contain antibodies to at least four additional substances according to the precipitation lines observed Crude or partially purified Wood 46 toxin had at least two additional substances whose antibodies were not found in commercial antitoxin Corresponding lines were found when antibacterial immune sera diffused against growing staphylococci, and the substances responsible for these lines are apparently bacterial group antigens not identical to carbohydrate A or antigen A (Jensen)

DISCUSSION

Investigations have shown that multiple lines occur on agar precipitation when extracts of *Staph aureus* diffuse against rabbit immune sera produced against formalin-killed staphylococci This confirms the information obtained from slide agglutination, that the antigenic equipment of *Staph aureus* is very complex Knowledge of the identity of some of these lines is slowly accumulating and it is important that isolated observations are correlated, and that the lines and the substances responsible for them are given adequate designations The main purpose of the present paper has therefore been to review the literature on agar precipitation lines in *Staph aureus* systems and to describe lines which have been relatively well defined

It is undoubtedly of great importance and advantage when using the agar precipitation method on *Staph aureus*, to base investigations on substances which are already *partially* known through other activities *e g* agglutinating power, enzymatic activity, or toxic property Not only can more information about the substances in question be obtained in this way but the identification of the corresponding line will also reduce the number of unknown lines and facilitate their identification
agglutinogens described by us, *three* can be recognized as easily re-
tion Our investigations have shown that of the "specific" *Staph aureus*

producible and characteristic lines on agar precipitation There may be several reasons why the other known agglutinogens have not been observed as precipitation lines

There are two main conditions necessary for the production of precipitation lines First, the antigen should be present in a soluble state, *i e* the agglutinogen has to be released from its possible attachment to the cell wall in a soluble form Second, the agar precipitation method requires higher concentrations of the antigen and the antibody than most other serological reactions Our observations with polysaccharide A (13) showed that the polysaccharide was released from the cell wall as a result of enzyme-autolysis of the wall When the enzyme was heat inactivated, no polysaccharide A line was obtained with whole or crushed bacteria It is reasonable to believe that, in most cases, the mere suspension of intact or disintegrated bacteria in saline or a buffer is inadequate for the liberation of the agglutinogens

It is more uncertain whether our factors are too weak for the production of precipitation lines Thus the *n* factor serum agglutinates strain 1503 to a dilution of 1 in 50 or 1 in 100 and yields a distinct line, whereas some other sera with agglutination titres up to 1 in 500 or 1 in 1,000 give no line In future work in this field, therefore, more stress should be laid upon the use of other extraction methods in order to release the agglutinogens in a soluble form

The agar precipitation method has already given important contributions to our knowledge of *Staph aureus* antigens and antibodies The method has given new data on antigens already recorded by other methods, and also revealed antigens and antibodies not described previously, and thereby stimulated research in this field A great advantage is its ability to distinguish different antigen-antibody systems present in a complex substance When working on the purification of antigens, the method is very valuable in that it gives information about quantitative variations in the antigen and on the presence of serologically active impurities Due to the high specificity of agar precipitation, minor molecular changes in an antigen, *e g* depolymerization or removal of labile groups, or minor variations in the antibody may result in a new line showing only partial or no coalescence with the original one Such findings may give important information as to the group responsible for the serological activity of a substance However, the high specificity also makes it difficult to decide whether each line represents a single antigen or has been produced through minor modifications in antigen or antibody It is, however, in our opinion, not likely that the agar precipitation method will be suitable for the routine serological typing of staphylococci The technique is more time consuming, and more sensitive to variations in antigen and antibody content than slide agglutination

It would be of great advantage if workers who observe lines in *Staph aureus* systems excluded the possibility that they may be identical to

previously described lines before publishing their results In our experience five of the *Staph aureus* lines are so well characterized that there should be no difficulty in recognizing them provided the reference system and the experimental conditions required for each are maintained One or more of these lines will undoubtedly be among the lines occurring on the plates when working with *Staph aureus* systems Other lines which have been described may be more difficult to produce, probably because the corresponding antibodies in immune sera and normal sera from animals are too weak Individual variations among the rabbits in the antibody response to staphylococcal materials have also to be taken into consideration The selection of suitable strains for immunization will probably reduce this difficulty

SUMMARY

Agar precipitation lines corresponding to agglutinogens h_1 and i_1 of *Staph aureus* have been described A line corresponding to the *n* agglutinogen has been described earlier Thus three of the "specific" agglutinogens used in our slide agglutination typing system have been recognized on agar precipitation Two group antigens of *Staph aureus*, polysaccharide A and antigen A (Jensen), are also easily recognized by characteristic agar lines Additional lines described in immune sera and normal sera are discussed

Reference systems and the characteristics of the lines are described It is hoped that this will help other workers to identify their lines

REFERENCES

1 *Bergdoll M S Surgalla M J & Dack G M* Staphylococcal enterotoxin Identification of a specific precipitating antibody with enterotoxin neutralizing property J Immunol 83 334–338 1959

2 *Butler L O* Studies on the preparation and isoelectric point of staphylococcal

3 *Co*

4 *Cohen J O Cowart G S & Cherry W B* Antibodies against *Staphylococcus aureus* in non immunized rabbits J Bact 82 110–114 1961

5 *Cohen J O & Oeding P* Serological typing of staphylococci by means of fluorescent antibodies 1 Development of specific reagents for seven serological factors J Bact 84 735–741 1962

6 *Fisk S D* Recognition of toxicogenic bacterial strains *in vitro* Brit M J 1 493 496 1948

7 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 1 Purification on DEAE cellulose columns Acta path et microbiol scandinav 55 110–116 1962

8 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 2 Further studies on purification methods Acta path et microbiol scandinav 55 117 126 1962

9 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 3 Some chemical and physicochemical properties Acta path et microbiol scandinav 55 289 298 1962

10 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 4 Paper chromatography of acid hydrolysates Acta path et micro biol scandinav 55 299–307 1962

11 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 5 Identification of amino sugars Quantitative determination of amino sugars and amino acids Acta path et microbiol scandinav 55 308 318 1962

12 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 6 Antigenic properties Acta path et microbiol scandinav 55 450–462 1962

13 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 7 On the chemical basis of the serological reactivity and the nature of the extraction process Acta path et microbiol scandinav 55 463–474 1962

14 *Haukenes G Ellwood D C Baddiley J & Oeding P* Serological cross reactivity between polysaccharide A and teichoic acid of *Staphylococcus aureus* Biochim et biophys acta 53 425–426 1961

15 *Haukenes G Losnegard N & Oeding P* Extraction and fractionation of antigenic material from *Staphylococcus aureus* Acta path et microbiol scandinav 53 84 94 1961

16 *Haukenes G & Oeding P* On two new antigens in *Staphylococcus aureus* Acta path et microbiol scandinav 49 237 248 1960

17 *Jensen K* A normally occurring staphylococcus antibody in human serum Acta path et microbiol scandinav 44 421 428 1958

18 *Jensen K* Undersøgelser over stafylococcernes antigenstruktur Thesis Munksgaard Copenhagen 1959

19 *Jensen K Serological typing of the staphylococci by means of gel precipitation* reactions Acta path et microbiol scandinav 52 175 184 1961

20 *Jensen K Neter E Gorzynski E A & Anzai H* Studies on toxic products of staphylococci Acta path et microbiol scandinav 53 191 200 1961

21 *Juli* ı

22 *Oedır*

23 *Oeding P* Staphylococcal antigen antibody reactions in agar Acta path et microbiol scandinav 47 53 64 1959

24 *Oeding P* Antigenic properties of *Staphylococcus aureus* Bact Rev 24 374 396 1960

25 *Stern H & Elek S D* Antigenic structure of *Staphylococcus pyogenes* J Path & Bact 73 473 483 1957

26 *Surgalla M J Bergdoll M S & Dack G M* Use of antigen antibody reactions in agar to follow the progress of fractionation of antigenic mixtures Application to purification of staphylococcal enterotoxin J Immunol 69 357 365 1952

27 *Turpin A Relyveld F H Pillet J & Raynaud M* Purification de la toxine et de l anatoxine staphylococciques alpha Ann Inst Pasteur 87 185 193 1954

28 *Woodin A M* Fractionation of a leucocidin from *Staphylococcus aureus* Biochem J 73 225 237 1959

FROM THE MARIA HOSPITAL DEPARTMENT OF MEDICINE (HEAD PROF G TÖTTERMAN M D) AND THE MICROBIOLOGICAL LABORATORY LÄÄKETEHDAS ORION OY HELSINKI

ANTIBODY RESPONSE TO PRIMARY TETANUS VACCINATION IN PATIENTS UNDER PROLONGED ANTICOAGULANT THERAPY

By

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Received 1.x 62

The site of antibody formation is not regarded as definitely established (4) But it is clear that a variety of cells in the general lymphoid tissue group can initiate the production of antibodies Likewise the reticulo endothelial system (RES) is involved in antibody formation Labelled antigens have been found in the reticulo endothelial cells and by the fluorescence technique antibody complexes have been detected in Kupffer s cells in the liver indicating an area of antibody formation (3)

In addition to antibody formation the functions of the reticulo endothelial system include the capacity of phagocytosing foreign bodies and the capacity of synthesizing clotting factors Various investigators have shown that proconvertin and prothrombin at any rate are synthesized in the RES (6 11 8 13 7 12) *Slatis* for instance reported a marked decrease of these clotting factors in the plasma after blockade of the reticulo-endothelial system in rats with Thorotrast Of the RES 70 to 90 per cent are situated in the liver (Kupffer s cells)

It is now generally agreed that four clotting factors are influenced by the administration of coumarin drugs namely prothrombin proconvertin factor IX and factor X The site of genesis of the last mentioned two factors has not been definitely established Proconvertin and prothrombin are formed in the RES Thus treatment with dicoumarol derivatives affects in part at least the capacity of the RES of synthesizing clotting factors

W[illegible]

cel[illegible] convincing evidence has been produced to the effect that prolonged treatment with dicoumarol or phenindione causes permanent injury to the liver (10 2 5) Only a few cases of obscure jaundice and sensitivity reactions complicating phenindione therapy have been reported (10 5) By contrast *Frelland & Jacobsen* (5) observed hepatitis in a strikingly large number of phenindione treated

10 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 4 Paper chromatography of acid hydrolysates Acta path et microbiol scandinav 55 299-307, 1962
11 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 5 Identification of amino sugars Quantitative determination of amino sugars and amino acids Acta path et microbiol scandinav 55 308 318 1962
12 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 6 Antigenic properties Acta path et microbiol scandinav 55 450 462, 1962
13 *Haukenes G* Immunochemical studies on polysaccharide A of *Staphylococcus aureus* 7 On the chemical basis of the serological reactivity and the nature of the extraction process Acta path et microbiol scandinav 55 463 474 1962
14 *Haukenes G Ellwood D C Baddiley J & Oeding P* Serological cross reactivity between polysaccharide A and teichoic acid of *Staphylococcus aureus* Biochim et biophys acta 53 425-426 1961
15 *Haukenes G Losnegard, N & Oeding P* Extraction and fractionation of anti [illegible] *us aureus* Acta path et microbiol scan
16 [illegible] antigens in *Staphylococcus aureus* Acta 237 248 1960
17 [illegible] phylococcus antibody in human serum Acta path et microbiol scandinav 44 421-428 1958
18 *Jensen K* Undersøgelser over stafylococcernes antigenstruktur Thesis Munksgaard Copenhagen 1959
19 *Jensen K* Serological typing of the staphylococci by means of gel precipitation reactions Acta path et microbiol scandinav 52 175 184 1961
20 *Jensen K Neter E Gorzynski E A & Anzai H* Studies on toxic products of [illegible] nav 53 191 200 1961
21 [illegible] pecificity of carbohydrates ol & Med 31 947 949 1934
22 [illegible] cci at various conditions [illegible] 1957
23 *Oeding P* Staphylococcal antigen antibody reactions in agar Acta path et microbiol scandinav 47 51 64 1959
24 *Oeding P* Antigenic properties of *Staphylococcus aureus* Bact Rev 24 374 396 1960
25 *Stern H & Fleck S D* Antigenic structure of *Staphylococcus pyogenes* J Path & Bact 73 473 483 1957
26 *Surgalla M J Bergdoll M S & Dack G M* Use of antigen antibody reactions in agar to follow the progress of fractionation of antigenic mixtures Application to purification of staphylococcal enterotoxin J Immunol 69 357-365 1952
27 *Turpin A Relyveld E H Pillet J & Raynaud M* Purification de la toxine et de l'anatoxine staphylococciques alpha Ann Inst Pasteur 87 185 193 1954
28 *Woodin A M* Fractionation of a leucocidin from *Staphylococcus aureus* Biochem J 73 225 237 1959

tetanus antitoxin (WHO Statens Seruminstitut Copenhagen Denmark) was included in each test

RESULTS

The tetanus antitoxin responses in the group of patients receiving anticoagulant therapy and in the control group are shown in Table 1 Although the mean antitoxin values were slightly higher in the control group than in the group of patients receiving anticoagulant therapy, the difference is not significant Of the 5 patients who had less than 0 03 units ml after vaccination, 3 were controls

TABLE 1

The Tetanus Antitoxin Responses in the Group Receiving Anticoagulant Therapy and in the Control Group Both Groups Were Immunized with two 5 Lf Doses of Adsorbed Tetanus Toxoid

Group	Number tested	Antitoxin units ml						
		0 03	0 03	0 1	0 3	1 0	Arithmetic mean	Geometric mean
Anticoagulant	28	2	6	8	10	2	0 216	0 114
Control	26	3	5	10	5	3	0 221	0 098

In the anticoagulant group the dispersion of responses was the same irrespective of the duration of the anticoagulant therapy (These patients had received anticoagulant therapy for periods varying between one month and 5 years)

The tetanus antitoxin responses in the group of patients receiving propylhydroxycoumarin seemed to be somewhat higher than the antitoxin responses of the other patients, who were given phenindione The series is too small however to allow of statistical comparisons between the antitoxin responses of the phenylpropylhydroxycoumarin- and the phenindione treated patients

DISCUSSION

The present study showed that the antitoxin responses to primary tetanus vaccination of patients on prolonged anticoagulant therapy were the same as of the controls No other definite conclusions are warranted however The results cannot be interpreted as evidence in favour of the view that prolonged anticoagulant therapy does not affect the antibody response in the RES since antibodies are also formed in the general lymphoid tissue group, which probably is not affected by anticoagulant therapy As early as 1949, *Oakley, Warrack & Batty* (9) *demonstrated in animal experiments* that antitoxin may be produced in the lymph glands draining the area injected with diphteria or tetanus A P T In the present study tetanus vaccine was *injected* subcutaneously into the arm Hence it is possible that antibody formation in part, at

patients These authors stated that the clinical picture as a rule was indicative of virus hepatitis and suggested that the disease was due to infection incurred in connection with the withdrawal of venous blood

The question arises whether the high incidence of virus hepatitis in patients on long-term phenindione therapy reported by *Fretland & Jacobsen* is due to the fact only that these patients are more exposed than others to infection owing to the mode of sample-taking or, whether anticoagulant treatment possibly lowers the resistance of the liver to infection by interfering with the phagocytosing function of the reticulo-endothelial system

In order to throw light on this problem *Adlercreutz et al* (1) administered colloidal Au^{198} intravenously to patients in prolonged anticoagulant therapy They found that the uptake in the liver often was poorer in the anticoagulant-treated patients than in the controls The uptake in the RES in the liver was particularly poor in patients who had received anticoagulant therapy for more than two months The uptake returned to normal after the completion of anticoagulant therapy The results reported by *Adlercreutz et al* seem to indicate that long-term anticoagulant therapy affects the phagocytosing capacity of the RES

Since the RES is involved in antibody formation it may be assumed that anticoagulant therapy, which affects the capacity of forming clotting factors and the phagocytosing capacity of this system, also affects antibody formation The present study was undertaken in order to investigate the antibody response to primary tetanus vaccination in patients on long-term anticoagulant therapy

MATERIAL AND METHODS

The patients The series consisted of 28 patients with cardiovascular disorders receiving continuous anticoagulant therapy and frequently visiting the Outpatients Clinic of the Maria Hospital They had not previously been vaccinated against tetanus

The anticoagulants used were phenindione (Trombosol) in 21 cases and phenylpropylhydroxycoumarin (Marcoumar) in 7 cases the average dose being *87 0 mg* (phenindione) and 2 77 mg (phenylpropylhydroxycoumarin) [1] The patients were from 36 to 77 years old the mean age being 54 years Most of the patients had a history of previous coronary occlusion

The control series consisted of 26 patients frequently visiting the Outpatients Clinic They had various cardiovascular and other disorders but a negative history of anticoagulant therapy and tetanus vaccination These patients were from 37 to 75 years old the mean age being 55 years

Vaccination Both groups received two doses of 0 5 ml (5 Lf)[2] of purified tetanus toxoid adsorbed on aluminium phosphate (Tetanus Vaccine Orion) subcutaneously with an interval of 4 weeks

Specimens Blood specimens were taken before vaccination and two weeks after the second vaccination The sera were stored at −25° C until the antitoxin determinations were carried out

Tetanus antitoxin determination The individual sera were titrated for tetanus antitoxin in mice weighing 18–20 grams at the level of 0 01 units/ml Standard

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1 The mean P-P time (Owren) was 29 3

2 5 Lf = 5 flocculation units

FROM THE DEPARTMENT OF CLINICAL BACTERIOLOGY UNIVERSITY OF LUND
GENERAL HOSPITAL MALMÖ SWEDEN

"NORMAL" VALUES OF ANTISTREPTOLYSIN AND ANTISTAPHYLOLYSIN TITRES AS STUDIED OVER $4^1/_2$ YEARS IN THE SERA OF HEALTHY BLOOD DONORS

By

RAGNAR INGESTAD and STEN WINBLAD

Received 31 x 62

Current serological methods have made it possible to assay quantitatively the group specific, and consequently not type-specific, antibodies antistreptolysin (AST) and anti alpha toxin (antistaphylolysin, ASta) when they are present in titres lower than those seen in connection with clinical infections with haemolytic streptococci or yellow staphylococci Since exposure to these bacteria is not infrequent, measurable amounts of antibodies against them may be found even in healthy individuals It is obviously important to determine the titres in a healthy population as these form the basis on which to decide which ones of the titres are to be regarded as 'elevated' and thus indicate of present or recent infection with haemolytic streptococci or yellow staphylococci In regard to AST both *Kalbak* and *Winblad*, who first studied these antibodies in Scandinavia considered AST titres above 200 units as indicative of fairly recent contact with, if not actually present, haemolytic streptococci This limit value was calculated with due regard to the titres found in healthy individuals As for the ASta titre, early investigations proved that values amounting to 2 IU ml occurred so often in healthy subjects that only titres above this level might be taken as possible signs of staphylococcal infections of any importance

Since then AST values in healthy persons have been extensively studied by various workers (3 8 10–12 17 18) As early as 1935, *Coburn & Pauli* (4) found that in USA healthy people showed geographical AST variations the titre diminishing from north to south

In 1949 *Schøne & Steen* (14, 16) presented a study comprising more than 500 healthy Norwegian blood donors Neither AST nor ASta were found to vary with sex or age In 95 per cent of the examined subjects AST was less than 200 and ASta less than 2 8 IU ml An ASta of more than 3 5 IU ml was extremely rare In this examination of a presumably normal Norwegian population positive correlation was found between a high AST titre and the presence of haemolytic streptococci in the

least, took place in the lymph glands, without involving the whole of the reticulo-endothelial system

The purpose of the present study was to investigate the antibody response during prolonged anticoagulant therapy The results are not directly comparable with those of *Adlercreutz et al*, who studied the phagocytosing capacity of the RES and observed that it was impaired during long-term anticoagulant treatment

It should be emphasized that from a functional standpoint the RES is not a static system, but undergoes considerable variations under various conditions of irritation This is a factor rendering more difficult the evaluation of the results of tolerance tests on the RES, *e g* in the form of tetanus vaccination during anticoagulant therapy

SUMMARY

The antibody response to subcutaneous primary tetanus vaccination was investigated in a series of 28 patients with cardiovascular disorders receiving long-term anticoagulant therapy in the form of phenindione or phenylpropylhydroxycoumarin and in a control group of 26 patients with cardiovascular and other disorders Although the mean values were slightly higher in the control group than in the group receiving anticoagulant therapy, the difference is not significant

Within the anticoagulant group the dispersion of the antitoxin responses was the same irrespective of the duration of anticoagulant therapy given prior to tetanus vaccination

REFERENCES

1 *Adlercreutz F & Pettersson T* The reticuloendothelial system and anticoagulant therapy as studied by radiogold clearence The Lancet *I* 470–471 1963
2 *Bjerkelund Chr J* The effect of long term treatment with dicoumarol in myocardial infarction Acta med scand *158* suppl 330 171 1957
3 *Coons A H & Kaplan M H* Localization of antigen in tissue cells J exp Med *91* 1–13 1950
4 *Criep Leo H* Clinical immunology and allergy Grune & Stratton New York and London 1962
5 *Fretland A & Jacobsen C D* Liver function in patients on long term treatment with anticoagulants Nord med *65* 324 331 1961
6 *Heppich E & Schmid J* Wien Zschr inn Med *29* 195 1948 Cit Schmid J 1951
7 ... der Prothrombin in Tierversuch Schweiz
8 ... prothrombina del sangue midollare con ... Hematologica 35 177 189 1951
9 ... *C H & Batty I* Sites of antibody production J Path
10 ... *L & Watt P J* Anticoagulants and the liver ... nt therapy Harvey & Blythe Ltd 1961
11 ... Theorie und Praxis Maudrich Verlag Wien 1951
12 *Slätis P* The synthesis of prothrombin and proconvertin in the reticulo endothelial system Scandin J Clin Lab Invest *10* suppl 33 1958
13 *Witte S & Dirnberger P* Über den Bildungsort der gerinnungsspezifischen Plasmaproteine Klin Wschr *31* 39 40 1953 b

FROM THE DEPARTMENT OF CLINICAL BACTERIOLOGY, UNIVERSITY OF LUND
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"NORMAL" VALUES OF ANTISTREPTOLYSIN AND ANTISTAPHYLOLYSIN TITRES AS STUDIED OVER $1\frac{1}{2}$ YEARS IN THE SERA OF HEALTHY BLOOD DONORS

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In 1949 *Schone & Steen* (11, 16) presented a study comprising more than 500 healthy Norwegian blood donors. Neither AST nor ASta were found to vary with sex or age. In 95 per cent of the examined subjects AST was less than 200 and ASta less than 2.8 IU/ml. An ASta of more than 3.5 IU ml was extremely rare. In this examination of a presumably normal Norwegian population positive correlation was found between a high AST titre and the presence of haemolytic streptococci in the

nose and throat, and correspondingly, between a high ASta titre and the presence of yellow staphylococci in the nose and throat

Kohler (8) in 1958 examined 600 inhabitants of Northern Germany who did not suffer from any streptococcal disease The mean AST value was independent of age up to sixty years, and apparently unrelated to sex and seasons of the year

On the basis of an undifferentiated pathological material, *Seifert* (13–15) studied AST titre variations as to age, sex, geographical latitude, and time of the year On the basis of 38,214 examinations he concluded that the AST titre reaches a maximum at the age of 6–10 years and gradually diminishes in later life By means of 46,632 examinations, Seifert could further demonstrate that AST titres were lowest in southern regions This observation is supported by the fact that Italian investigators (1, 3, 5, 12) have reported strikingly low mean values of AST AST investigations on 164,438 individuals (67,893 men and 64,455 women) undertaken in the years 1956–59 showed AST titres to reach their maximum in the winter The average value was higher for men than for women

It seems desirable, for the reasons stated above, to investigate for several years the titres of the two antibodies in healthy subjects, in this case blood donors, with special regard to seasonal variation These examinations were performed in one laboratory, the same working staff and technique being used throughout, as well as the same antitoxic standard to estimate the respective strength of streptolysin and staphylolysin Under these conditions the results obtained should be of considerable reliability

MATERIAL AND METHODS

The AST and ASta examinations have been made during 1957–1961 on 1422 subjectively healthy persons (blood donors) living in Malmo Sweden Their age and sex distributions are given in Table 1

TABLE 1
Age and Sex Distribution

Age	Men N	Men %	Women N	Women %
< 20	31	2 6	6	2 5
20 24	116	9 8	28	11 8
25 29	204	17 2	33	13 9
30 34	244	20 6	42	17 6
35–39	216	18 2	46	19 3
40 44	189	16 0	34	14 3
45 49	114	9 6	31	13 0
50–54	46	3 9	15	6 3
55 59	16	1 4	2	0 8
60 64	8	0 7	1	0 4
	1184	100 0	238	99 9
Median	34 years		36 years	

In order to be accepted as a blood donor the applicant is subjected to Hb, ESR Wassermann and pulmonary X ray examinations the first three of these procedures are repeated at each blood donation The AST and ASta examinations have been made mainly on sera withdrawn for such procedures 2046 AST, and 778 ASta analyses have been carried out, and as a consequence some of the donors have, by chance been analyzed more than once during the relevant 5 year period

Antistreptolysin reaction Streptolysin from a broth culture of the S 84 strain is used The broth for streptolysin production is made on ox heart with 2 per cent peptone 0 2 per cent glucose, 0 2 per cent $NaHCO_3$ and 0 1 per cent Na_2HPO_4 with pH adjusted to 8 0 The streptolysin broth is centrifuged and filtered through a Seitz filter The amount of streptolysin units is determined by titration against standard antistreptolysin serum after reduction by a 0 4 per cent sodium pyrosulphite solution The standard antistreptolysin globulin is obtained from the Danish State Serum Institute of Copenhagen

A suitable amount of streptolysin broth containing 1 unit/ml is reduced by a 0 4 per cent sodium pyrosulphite solution The patient s serum is diluted with normal saline in small tubes to a total volume of 1 ml per tube and is inactivated 0 5 ml ... ibation of this mixture in a 37° C ... t suspension of sheep erythrocytes ... C for 30 minutes and allowed to ... amount of haemolysis is determined ... units present is derived from the number of streptolysin units inactivated

Antistaphylolysin reaction Staphylococcal toxin is obtained from the State ...

... further incubation for 1 hour at 37° C, followed by overnight incubation at 4° C, the amount of antitoxin units which has been neutralized is determined

Statistical analysis Owing to the uneven distributions and the properties of the scales ... atistical analysis o' ... onal aid with these c... the common mediar ... χ^2 test (4)

$$\chi^2 = \sum_{i=1}^{r} \sum_{j=1}^{k} \frac{(O_{ij} - E_{ij})^2}{E_{ij}}$$

RESULTS

Distribution of titre values 63 3 per cent of all the observed AST titres were below 200 units, and 81 3 per cent below 300 units Titres equal to or above 300 antistreptolysin units are consequently encountered in 18 7 per cent of the cases

Correspondingly, 58 5 per cent of the ASta titres of the examined sera were below 2 0 units, and 79 0 per cent below 3 0 antistaphylolysin units which leaves 21 per cent at or above 3 0

Monthly and early variations of titre values Tables 2 and 3 show the results of the AST analyses after they have been arranged according to month and year and also show the medians of the material both when thus arranged and when taken as a whole The seasonal fluctuations of the AST titres are illustrated in Fig 1 where the two curves which are based on the percentage distribution of these values, represent AST values equal to or above 300 IU ml and 200 IU ml respectively

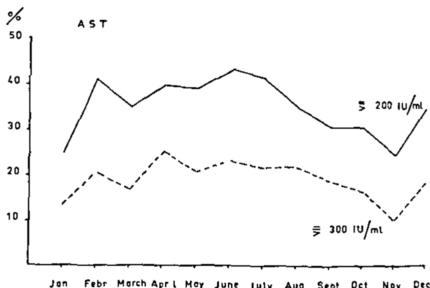


Fig 1
AST titres seasonal variation

It is clear from this figure that during the period from February to July there is a plateau of comparatively high AST values, followed by a decline with a minimum in November after which there is an increase until February This general trend repeats itself each year but it is best seen from a representation of the whole material where each month is taken separately When the medians of the AST values of the years 1957–1961 are compared, there is no significant difference from one year to another The distribution of AST titres in healthy subjects seems to be largely the same during all the years under investigation

A similar analysis of the ASta values during a 5-year-period yielded

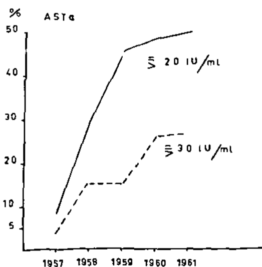


Fig 2
ASta titres variations from 1957–1961

TABLE 2

AST Titres Monthly Distribution

	0-49	50-99	100-149	150-199	200-249	250-299	300-349	350-399	400-449	450-499	500	Total	Median
January	12	40	28	40	7	12	4	7	4	0	6	160	149 5
February	5	26	36	35	16	20	8	3	3	0	22	174	178 1
March	11	44	18	40	16	14	8	10	3	0	9	173	166 4
April	3	16	22	24	6	11	9	10	3	0	5	109	177 6
May	7	33	22	22	14	11	3	9	4	0	13	138	165 4
June	6	23	24	29	15	14	7	7	2	0	17	144	182 3
July	10	27	26	42	18	18	11	7	8	0	14	181	182 2
August	8	38	29	44	13	12	7	12	3	0	18	184	168 8
September	12	39	35	42	14	8	13	5	3	0	15	186	157 8
October	14	40	32	40	9	16	6	7	6	0	12	182	155 8
November	20	48	43	52	11	21	5	5	5	0	6	216	146 0
December	21	33	36	39	17	14	12	9	6	0	12	199	161 7
	129	407	351	449	156	171	93	91	50	0	149	2046	164 6

$\chi^2 = 31.13$ df = 11 $P < .01$

AST Titres Yearly Distribution

TABLE 3

	0-49	50-99	100-149	150-199	200-249	250-299	300-349	350-399	400-449	450-499	500	Total	Median
1957	17	19	20	49	5	20	8	11	3	0	21	176	182 2
1958	20	39	32	43	11	14	12	9	3	0	23	206	163 5
1959	38	107	85	101	47	44	25	19	6	0	47	519	164 1
1960	22	131	96	139	43	50	24	26	18	0	29	578	163 9
1961	32	111	118	117	50	40	24	26	20	0	29	567	159 1
	129	407	351	449	156	171	93	91	50	0	149	2046	164 6

$\chi^2 = 4.34$ df = 4 $P > .30$

TABLE 4

ASta Titres Monthly Distribution

	0–0.9	1.0–1.9*		2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	6.0–6.9	7.0–7.9	8.0–8.9	9.0–9.9	10.0	Total	Median
		1.0–1.4	1.5–1.9											
January	11	16	4	18	6	1	4	0	1	0	0	0	61	1.89
February	12	16	4	16	5	5	7	1	0	0	0	1	67	2.04
March	11	18	2	15	5	0	7	0	2	0	0	0	60	1.70
April	10	15	4	10	1	1	7	0	0	0	0	2	50	1.45
May	18	18	3	11	2	1	1	0	2	0	0	1	57	1.21
June	14	10	2	5	3	1	1	0	1	0	0	2	39	1.23
July	24	12	5	6	5	2	4	0	0	1	0	2	61	1.22
August	19	22	4	16	9	1	2	0	1	1	0	0	75	1.37
September	29	16	6	16	2	3	7	1	0	1	0	2	83	1.34
October	19	14	9	16	8	5	2	1	2	1	0	2	79	1.81
November	22	18	6	18	4	1	5	0	1	1	0	2	78	1.42
December	18	19	5	13	4	1	6	0	0	1	0	1	68	1.37
	207	194	54	160	54	22	53	3	10	6	0	15	778	1.42

$\chi = 13.82$ df = 11 P > .20

* Values within each class deviate considerably. To get a more correct median, the 1.0–1.9 class has been split in half. However the *deviation is not completely compensated for by this means.*

TABLE 5

ASla Titres Yearly Distribution

	0-0 9	1 0-1 9*		2 0-2 9	3 0-3 9	4 0-4 9	5 0-5 9	6 0-6 9	7 0-7 9	8 0-8 9	9 0-9 9	+ 10 0	Total	Median
		1 0-1 4	1 5-1 9											
1957	61	17	3	4	2	0	1	0	0	0	0	0	88	0 90
1958	29	27	12	14	4	1	6	0	0	0	0	4	97	1 31
1959	12	30	9	29	6	3	3	0	0	2	0	0	94	1 73
1960	46	61	15	51	22	8	21	1	2	2	0	4	233	1 77
1961	59	59	15	62	20	10	22	2	8	2	0	7	266	1 95
	207	194	54	160	54	22	53	3	10	6	0	15	778	1 42

$\chi^2 = 60.04$ df = 4 $P < .001$

* Values within each class deviate considerably To get a more correct median the "1 0 1 9" class has been split in half However, the deviation is not completely compensated for by this means

TABLE 4

ASta Titres Monthly Distribution

	0-0.9	1.0-1.9*		2.0-2.9	3.0-3.9	4.0-4.9	5.0-5.9	6.0-6.9	7.0-7.9	8.0-8.9	9.0-9.9	10.0	Total	Median
		1.0-1.4	1.5-1.9											
January	11	16	4	18	6	1	4	0	1	0	0	0	61	1.89
February	12	16	4	16	5	5	7	1	0	0	0	1	67	2.04
March	11	18	2	15	5	0	7	0	2	0	0	0	60	1.70
April	10	15	4	10	1	1	7	0	0	0	0	2	50	1.45
May	18	18	3	11	2	1	1	0	2	0	0	1	57	1.21
June	14	10	2	5	3	1	1	0	1	0	0	2	39	1.23
July	24	12	5	6	5	2	4	0	0	1	0	2	61	1.22
August	19	22	4	16	9	1	2	0	1	1	0	0	75	1.37
September	29	16	6	16	2	3	7	1	0	1	0	2	83	1.34
October	19	14	9	16	8	5	2	1	2	1	0	2	79	1.81
November	22	18	6	18	4	1	5	0	1	1	0	2	78	1.42
December	18	19	5	13	4	1	6	0	0	1	0	1	68	1.37
	207	134	54	160	54	22	53	3	10	6	0	15	778	1.42

$\chi^2 = 13.82$ df = 11 $P > .20$

* Values within each class deviate considerably. To get a more correct median the 1.0-1.9 class has been split in half. However the deviation is not completely compensated for by this means.

TABLE 5

ASta Titres Yearly Distribution

	0-0.9	1.0-1.9*		2.0-2.9	3.0-3.9	4.0-4.9	5.0-5.9	6.0-6.9	7.0-7.9	8.0-8.9	9.0-9.9	≥10.0	Total	Median
		1.0-1.4	1.5-1.9											
1957	61	17	3	4	2	0	1	0	0	0	0	0	88	0.90
1958	29	27	12	14	4	1	6	0	0	0	0	4	97	1.31
1959	12	30	[illegible]	29	6	3	3	0	0	2	0	0	94	1.73
1960	46	61	15	51	22	8	21	1	2	2	0	4	233	1.77
1961	59	59	15	62	20	10	22	2	8	2	0	7	266	1.95
	207	194	54	160	54	22	53	3	10	6	0	15	778	1.42

$\chi^2 = 60.04$ df = 4 $P < .001$

* Values within each class deviate considerably. To get a more correct median the 1.0-1.9 class has been split in half. However the deviation is not completely compensated for by this means.

TABLE 4

ASta Titres Monthly Distribution

	0-0.9	1.0-1.9*		2.0-2.9	3.0-3.9	4.0-4.9	5.0-5.9	6.0-6.9	7.0-7.9	8.0-8.9	9.0-9.9	10.0	Total	Median
		1.0-1.4	1.5-1.9											
January	11	16	4	18	6	1	4	0	1	0	0	0	61	1.89
February	12	16	4	16	5	5	7	1	0	0	0	1	67	2.04
March	11	18	2	15	5	0	7	0	2	0	0	0	60	1.70
April	10	15	4	10	1	1	7	0	0	0	0	2	50	1.45
May	18	18	3	11	2	1	1	0	2	0	0	1	57	1.21
June	14	10	2	5	3	1	1	0	1	0	0	2	39	1.23
July	24	12	5	6	5	2	4	0	0	1	0	2	61	1.22
August	19	22	4	16	9	1	2	0	1	1	0	0	75	1.37
September	20	16	6	16	2	3	7	1	0	1	0	2	83	1.34
October	19	14	9	16	8	5	2	1	2	1	0	2	79	1.81
November	22	18	6	18	4	1	5	0	1	1	0	2	78	1.42
December	18	19	5	13	4	1	6	0	0	1	0	1	68	1.37
	207	194	54	*160*	54	*22*	53	*3*	*10*	*6*	*0*	15	778	*1.42*

χ = 13.82 df = 11 P > .20

* Values within each class deviate considerably. To get a more correct median the 1.0-1.9 class has been split in half. However the deviation is not completely compensated for by this means.

TABLE 5

ASla Titres Yearly Distribution

	0-0 9	1 0-1 9*		2 0-2 9	3 0-3 9	4 0-4 9	5 0-5 9	6 0-6 9	7 0-7 9	8 0-8 9	9 0-9 9	10 0	Total	Median
		1 0-1 4	1 5-1 9											
1957	61	17	3	4	2	0	1	0	0	0	0	0	88	0 90
1958	29	27	12	14	4	1	6	0	0	0	0	4	97	1 31
1959	12	30	9	29	6	3	3	0	0	2	0	0	94	1 73
1960	46	61	15	51	22	8	21	1	2	2	0	4	233	1 77
1961	59	59	15	62	20	10	22	2	8	2	0	7	266	1 95
	207	194	54	160	54	22	53	3	10	6	0	15	778	1 42

$\chi^2 = 60{,}04$ df = 4 P < 001

* Values within each class deviate considerably To get a more correct median the 1 0 1 9 class has been split in half However, the deviation is not completely compensated for by this means

TABLE 4

ASta Titres Monthly Distribution

	0-0.9	1.0-1.9*		2.0-2.9	3.0-3.9	4.0-4.9	5.0-5.9	6.0-6.9	7.0-7.9	8.0-8.9	9.0-9.9	10.0	Total	Median
		1.0-1.4	1.5-1.9											
January	11	16	4	18	6	1	4	0	1	0	0	0	61	1.89
February	12	16	4	16	5	5	7	1	0	0	0	1	67	2.04
March	11	18	2	15	5	0	7	0	2	0	0	0	60	1.70
April	10	15	4	10	1	1	7	0	0	0	0	2	50	1.45
May	18	18	3	11	2	1	1	0	2	0	0	1	57	1.24
June	14	10	2	5	3	1	1	0	1	0	0	2	39	1.23
July	24	12	5	6	5	2	4	0	0	1	0	2	61	1.22
August	19	22	4	16	9	1	2	0	1	1	0	0	75	1.37
September	29	16	6	16	2	3	7	1	0	1	0	2	83	1.34
October	19	14	9	16	8	5	2	1	2	1	0	2	79	1.81
November	22	18	6	18	4	1	5	0	1	1	0	2	78	1.42
December	18	19	5	13	4	1	6	0	0	1	0	1	68	1.37
	207	194	54	100	54	22	53	3	10	6	0	15	778	1.42

$\chi^2 = 13.82$ $df = 11$ $P > .20$

* Values within each class deviate considerably. To get a more correct median the 1.0-1.9 class has been split in half. However the deviation is not completely compensated for by this means.

The constant yearly AST medians of each of the five years are good evidence of the likewise constant methodological conditions More in direct proof of the reliability of the AST technique used is furnished by the clinical observation that during this five-year-period there has been no increase of infections caused by haemolytic streptococci either

The ASta titres, on the other hand, did not change significantly with the time of the year If Staphylococcus aureus does play a rôle, whether primary or secondary, in the origin of the so called family infections this influence is not appreciably reflected in the ASta titre curves

It must be emphasized however, that the antistaphylolysin titre medians obtained from this population are by no means continously on the same level, on the contrary, they increase gradually from 1957–1961 This increase is paralleled by the growing frequency of infections with Staphylococcus aureus which has been demonstrated especially in hospital wards during the relevant period These observations also reflect the wide spread interest in infections of this kind which has characterized these last five years Research in this field has shown conclusively that these infections are caused by strains that have been selected by being resistant to penicillin It is obviously a matter of some difficulty to establish whether infections with Staphylococcus aureus are more frequent now than before The only supplementary proof of this has been found in large blood culture materials *e g* at the Danish State Serum Institute where Staphylococcus aureus has passed from a non dominating to a clearly dominating position This might indicate that infections with Staphylococcus aureus are more common today than they used to be Such a development would explain satisfactorily the steady rise of the antibody titres from 1957 to 1961 in "healthy' blood donors as they would then be more often exposed to pathogenic Staphylococcus aureus Furthermore, the possibility that selected staphylococci may have acquired a greater pathogenicity should not be totally disregarded since such strains might produce stronger antibody responses

Previous investigations have shown that basal carriers of Staphylococcus aureus generally have higher ASta titres than non carriers (*Packalen & Bergqvist*, 9) At the onset of the present investigation the frequency of such carriers among the blood donors seemed more or less unrelated to the matter at issue, and was not looked into *As things* turned out this proved an unfortunate oversight which hardly could have been avoided however in view of the original aim of this work a routine control examination of ASta The presence of an increased number of carriers of staphylococci in these years would imply a likewise increased exposure to Staphylococcus aureus A new investigation of "normal ASta titres in a few years' time and in the same geographical region should throw further light on this subject

the results presented in Tables 4 and 5 and in graphical form in Fig 2 As opposed to AST, the ASta titres do not vary appreciably with the time of the year It is true that in October, January, February, and March the mean value is higher than the one occurring during the rest of the year, but since the difference is not significant it may well be accidental On the other hand, there is a striking increase of high ASta percentage from 1957 to 1961

In view of the fact that one international standard was used throughout, the observation that AST values change significantly with the time of the year, and ASta values with different years, must decidedly be considered to be relevant

DISCUSSION

For clinical use, a single antibody titre reading is of no real diagnostic value unless it is extremely high or extremely low It follows, that the development of antibodies should be determined by repeated titrations If, however, a "limit value" could be defined, this would obviously be of help although at the same time, its limitations must be clearly realized and kept in mind

Up to now, the limit value of AST has been generally accepted to be 200 IU/ml but a slight modification is obviously called for at this point Judging from the findings here reported, AST titres ranging from 200 to 300 IU/ml are so common in a healthy, urban, Swedish population examined that only values of 300 units or above should be considered sufficiently high to indicate a recent or actually present infection with haemolytic streptococci Similarly, in the case of ASta, only values of 3 0 IU/ml or more are sufficient proof that the patient has, or has just had, an infection with Staphylococcus aureus On this assumption it may thus be concluded that some 20 per cent of the healthy population the Malmo area have either AST or ASta values above normal

It is clear from the analyses that AST titres are characterized by significant variations according to the time of the year but remains relatively constant year to year The AST median is highest in the spring and early summer, declines in the autumn and does not really start to increase again until after midwinter Infections with haemolytic streptococci belong to the group of "family infections" and these are largely passed on from one school-child to another In Sweden, June, July, and August are the months of school-vacation, and family infections have long been known to start in the autumn term and continue, throughout winter and spring The antibody responses to such infections are to be expected after a latency of one or two months In concordance with this the antibody curve rendered here does not coincide exactly in time with the curve of family infections but is, as it were, a couple of months late This appears to be the most likely explanation of the seasonal variations that were found to exist

FROM RICKETTSIA & VIRUS DEPARTMENT, STATENS SERUMINSTITUT, COPENHAGEN

STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

2. *Treatment of Virus Carrier Mice by Adoptive Immunization*

By

MOGENS VOLKERT

Received 8.xi.62

In the preliminary report (17) a description was given of experiments which showed for the first time that transplantation of isologous immune lymphoid cells to Lymphocytic Choriomeningitis (LCM) virus carrier mice caused a marked reduction of the virus titres in the blood and spleen. In some cases it even appeared that almost complete removal of the virus from the virus carriers had been achieved. As these results are in some ways similar to what is seen when transplantation of immune lymphoid cells is used to terminate a tolerant state to tissue grafts, they give new support for the assumption of the existence of an immunological tolerance to the LCM virus in mice.

The question of immunological tolerance to a virus is of more than theoretical interest. Firstly, if a tolerant state to one virus really does exist, this would point to the possibility of tolerance to other viruses and furthermore to the question of whether the tolerant phenomenon might not play a rôle in certain inapparant virus infections. Secondly, if a LCM virus carrier state can be brought to an end by experimental procedures, it seems adequate to consider the possibility of terminating other virus carrier states, e.g. to tumor viruses and to the many viruses which are isolated in great numbers from apparently healthy cells, animals and human beings all over the world.

Thirdly, it can be mentioned that when a virus carrier state develops because of tolerance, titration of the virus in the blood and organs will bring into existence a new possibility for measuring the effect of certain procedures which influence the tolerant state. Animals tolerant to a virus might therefore be valuable for immunological studies.

For these reasons further studies on tolerance to the LCM virus have been carried out. It is the purpose of this article to describe in more detail the results obtained when isologous lymphoid cells are transplanted to virus carrier mice.

The author is indebted to Mr. *Weis Bent*, chief of the biostatistic department at Statens Seruminstitut, for help in evaluating the experimental results, and to Miss *B. Hejer* for skilled technical assistance.

SUMMARY

The AST and ASta titres in blood-donors' sera were studied continuously over a period of 5 years. Altogether, 2046 serum specimens were examined for AST and 778 specimens for ASta. Of these, 81.3 per cent showed AST titres below 300 units, and 79.0 per cent ASta titres below 3.0 units. AST values of 300 units or more and ASta values of 3.0 units or more can therefore be regarded as "elevated".

The percentage of blood-donors' sera with AST titres of 300 units or above was at its lowest in September, October, and November, but remained unchanged from 1957 to 1961.

The percentage of blood-donors' sera with elevated ASta titres showed no change during the months of the year. In contrast, this percentage showed a definite increase from 1957 to 1961. The reasons for this are discussed.

REFERENCES

1 *Baduali C., Bugiardini G., Venturi R. & Zoli A.* The antistaphylolysin α content of the serum in healthy subjects and in subjects affected with diseases of staphylococcal and of non staphylococcal aetiology. Excerpt Med Microbiol *11* 45 1958
2 *Brown C. W. & Mood A. M.* Proceedings of the second Berkeley symposium on mathematical statistics and probability. P. 159 1951
3 *Bugiardini G. & Ferrucci M.* A study of the antistreptolysin titre in healthy subjects and in patients with different diseases. Excerpta Med Microbiol *9* 736 1955
4 *Coburn A. F. & Pauli R.* Limited observations on the antistreptolysin titre in relation to latitude. J Imm *29* 515 1935
5 *In erillo R., Azzena D. & Ma ucco C.* The reaction of the anti O streptolysin in normal subjects and in some pathological conditions. Excerpt Med Microbiol *10* 688 1957
6 *Jessen O., Faber V. & Rosendahl K.* Hyppigheden af forskellige bakteriemifor mer. Ugeskr for læger p 1613 1959
7 *Kalbak K.* Undersøgelse over O streptolysin og forekomsten af O antistreptolysin i serum. Munksgaard København 1942
8 *Kohler W.* Der Antistreptolysin Normaltiter in NO Deutschland. Zeitschr Imm forsch *116* 145 1959
9 *Packalen Th. & Bergqvist S.* Staphylococci in throat and nose and antistaphylolysin titre. Acta med scandinav *127* 291 1947
10 *Saint Martin M.* Antistreptolysin O determination in health and in disease. Canad med Assoc J *76* 627 1957
11 *Schone R. & Steen E.* Antistreptolysintiter hos friske. Nord Med *42* 1863 1949
12 *Seghini G.* The antistreptolysin titre in healthy subjects. Excerpt Med Microbiol *10* 678 1957
13 *Seifert H.* Antistreptolysin Titer und Lebensalter. Zeitschr f d ges inn Med *14* 641 1959
14 *Seifert H.* Serum Antistreptolysintiter und geographische Breitenlage. Zeitschr f d ges inn Med *14* 1133 1959
15 *Seifert H.* Über die Saisongabhangigkeit und Geschlechtgebundenheit des menschlichen Serum Antistreptolysin. Zeitschr f d ges inn Med *15* 589 1960
16 *Steen E. & Schone R.* Antistafylolysinreaksjonen. Nord Med *42* 1294 1949
17 *Westergren A.* Om normalvardet for sankningsreaktionen samt vissa serologiska titra (AS, ASta, Coliaggl.). Nord Med *42* 1290 1949
18 *Winblad S.* Studies in haemolytic streptococcus fibrinolysin, antifibrinolysin and antistreptolysin. Acta path et microbiol scandinav Suppl *44* 1941

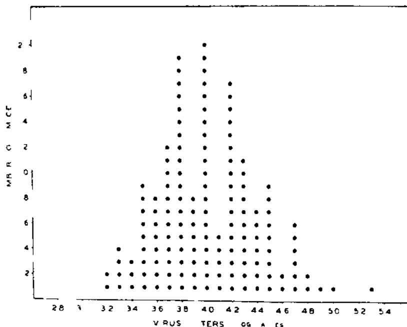


Fig. 1
The blood virus titres of 147 untreated virus carriers

types of blood will be discussed together in the experiments described. However only titres which were lower than any seen in untreated virus carriers in heart as well as tail blood will be considered as being significantly reduced, i.e. only titres below 10 6

Virus titres of the spleens of untreated virus carriers. Together with the virus titres of the blood the titres of the spleens were used to evaluate the effect of the adoptive immunization procedures on the virus carriers. An estimate of the virus titres of the spleens of untreated virus carriers was therefore attempted. For obvious reasons the number of such spleens available has been rather limited. In all 53 spleens from untreated mice have been titrated. The majority of these came from the mice in the various control groups when these animals were killed ...

... as having been reduced by the treatment given ... consider only titres lower than

The cell suspensions for transplantation were mixtures of cells ... lymph ...

ber ...

that ... narrowest possible range. Usually the mice were not younger than six weeks and not older than eight weeks. All the mice were then bled from their tails and a virus titration was carried out for each individual mouse. Shortly after the blood was taken the mice were divided into groups for the experiments by random selection. In all series of experiments one of these groups was kept as control and received no treatment but blood was tested for virus at the same time as in the transplanted groups.

Unless otherwise mentioned in the text all transplantations were carried out between animals of the same sex and by intravenous inoculation.

MATERIAL AND METHODS

The LCM virus used was provided by *Dr Traub* (Germany) and kept as described in the previous paper

The mice used as virus carriers and as donors were all, except when otherwise indicated in the text *from the same highly inbred strain of AkA mice* This strain is a Danish subline of AkR mice As in the case of these mice our strain has a high incidence of spontaneous leukemia but we have not observed any symptoms of this disease before the age of five to six months To avoid *any possible complication of* our results due to the leukemic disease no donor which was more than five months old was used nor were the cells harvested unless the organs on gross examination were found to be free of pathological lesions The recipients were likewise used when young usually at the age of six to eight weeks and in all cases the experiments were terminated before the mice reached the age of five months

Blood for virus titrations was taken from the tail vein by a calibrated micro pipette and from the heart by a syringe Before the samples were taken the pipettes or syringes were moistened with just enough heparin to prevent the blood from clotting during the first few minutes

The virus titrations were carried out by intracerebral inoculations in the institute's stock of ordinary white Swiss mice At the time of use their weight was between 12 and 14 grammes The titration endpoints were calculated according to the method of *Reed & Muench (14) In the figures* and tables presented logarithmic values are used throughout

The virus carrier state was brought about as described previously (17) by in*fecting newborn baby mice with the LCM virus In all cases the babies were in*oculated within the first 18 hours of life with 10-10(LD 50 doses of virus intra peritoneally In order to exclude any other foreign antigen than the virus antigen virus passed several times in AkA mice was used throughout Within the first two to three weeks after the inoculation 20–25 per cent of the babies died and many looked sick but after that time the remainder recovered and developed normally However all without exception were from now on virus carriers

Virus titres of the blood of untreated virus carriers To evaluate the effect of any treatment on virus carriers it is necessary to know the titre variation which can be expected within a group of untreated mice and within each individual mouse through the experimental observation time used For this purpose a complete titration was carried out on tail blood from 147 virus carriers at the age at which they were usually used in the main experiments *i e* six to seven weeks old The distribution of the titres in this group *is shown in Figure 1 It is seen from the figure that the* mean titre is 10^4 and that the variation from this titre is roughly $\pm 10^1$ None of the mice in this group had a titre lower than $10^{3.2}$ Moreover in the course of our

mice at ages of from six weeks to six months

s content of the blood determined in dilutions

tested in this way Only four mice had titres

10 9

from their tail

period of six weeks The mean variation of the virus titre in the *blood* of any individual mouse was found to be $10^{0.9}$ the greatest variation seen was $10^{2.4}$ and the least $10^{0.2}$ None of the mice showed titres lower than 10^3

As at the end of the *tolerance experiments heart blood* was *titrated for* virus instead of tail blood a comparison of the virus in tail blood and heart blood was carried out Nineteen virus carriers were bled first from their tails and thereafter from their hearts *and* the blood was titrated for virus One mouse showed a heart blood titre of 10-9 all other titration values were higher The difference in titres between the two types of blood varied from $10^{0.1}$ to $10^{1.3}$ and the mean titre for heart *blood* was $10^{0.3}$ lower than for tail blood

In the course of the experiments 53 untreated virus carriers in the control groups were bled from the heart and the blood was titrated for virus The mean titre was $10^{3.8}$ Six mice had titres below 10^3 but none was lower than $10^{2.6}$ These results indicate also that heart blood titres are *slightly lower than the titres found in* tail blood

Because of the small difference between the titres of heart and tail blood the two

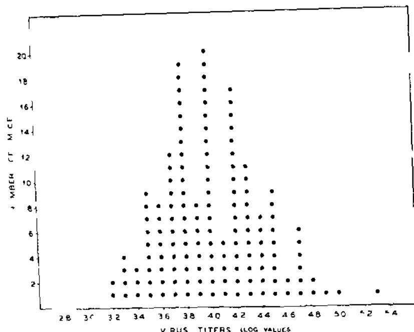


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[illegible]

... chosen to consider only titres lower than ... as having been reduced by the treatment given

The cell suspensions for transplantation were mixtures of cells from spleen and lymph nodes prepared as described in the previous paper

General experimental conditions For each series of experiments a suitable number of virus carriers was chosen The mice were selected according to sex and so that the ages of the mice were within the narrowest possible range Usually the mice were not younger than six weeks and not older than eight weeks All the mice were then bled from their tails and a virus titration was carried out for each individual mouse Shortly after the blood was taken the mice ... divided into groups for the experiment ... ents one of these groups was ... was tested for virus at th ...

Unless other... all transplantations were carried out between animals of the same sex and by intravenous inoculation

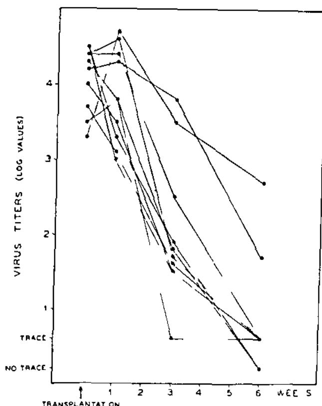


Fig 2

The blood virus titre curves for 10 virus carriers transplanted with 100×10^6 immune lymphoid cells

EXPERIMENTAL

The Effect of Transplantation of Isologous Lymphoid Cells from Immunized Mothers to Infected Babies

In the first series of experiments the mothers of the infected babies served as donors. As described in the previous paper, mothers of virus carrier babies are regularly infected by their offspring. Usually these infections run a symptomless course and end in solid immunity. In the preliminary report it has been shown that such mothers are a good source of immune lymphoid cells. However, to ensure that all the donors were immune, and to boost their immunity, in the following experiments 100 LD 50 doses of virus was given intraperitoneally at the time the mothers were taken away from their babies, *i.e.* four weeks after the babies were born. Two weeks after the booster dose of virus was given the mice were killed and the spleen and lymph node cells harvested and prepared for transplantation. In the course of eight months five groups of virus carriers were transplanted with this type of cells. In all, 46 virus carriers were injected with the immune lymphoid cells and all recipient mice received 100×10^6 cells. The virus titre in the tail blood was tested for each individual mouse at different inter-

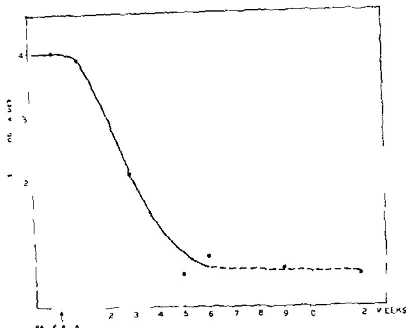


Fig 3
The mean bl d virus titre curve for 46 virus carriers transplanted with 100 × 10⁶ immune lymphoid cells

vals all in dilutions of blood from 10^{-1} to 10^{-3} At the end of the experiment the mice were killed and the virus titre in the heart blood and spleens determined Figure 2 shows the individual virus titres in the blood of a group of ten mice grafted with the same batch of cells and followed through six weeks It is clearly apparent that in the first week after the transplantation very little if anything happens to the virus the mean titre is at that time still about 10^4 However during the following two to three weeks there is a sharp decline in titre The figure also shows the variation of the transplantation effect on the virus from mouse to mouse This variation is most pronounced during the third week after the transplantation At that time most of the mice had virus titres of about 10 but one mouse had only traces of virus and another had a titre as high as $10^{3.8}$ Six weeks after the transplantation eight of the ten mice showed only traces of virus in blood diluted 1/10 but the remaining two still had quite high titres However even for these two a marked decline in titre is apparent As in all experiments a control group of untreated virus carriers was followed through the experimental period of six weeks None of these mice showed titres in their blood lower than $10^{3.3}$ and the mean titre was $10^{3.9}$

The results of the transplantation of the other four groups of mice were very similar to the one just described These mice, however were followed for a longer period after the transplantations Two of the

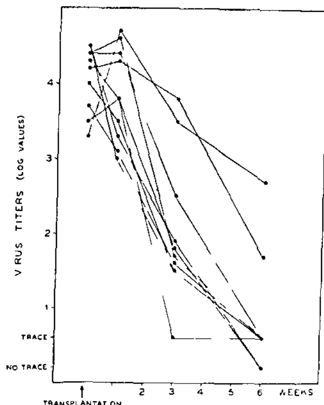


Fig 2
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the control groups killed at the same time are also shown in the table It is clearly apparent that within the first six weeks a very great reduction of the spleen titres occurs The difference between titres of some mice in the transplanted group and any mouse in the control group was more than 10^4 fold and the mean reduction more than 10^3 fold After 12 weeks the difference between the two groups is even more pronounced Four out of the fourteen transplanted mice did not have any detectable virus in the 10 per cent spleen suspension tested However, three of the four mice with no detectable virus belonged to the same group of seven mice, and this effect may be unusual

TABLE 2
Virus Titres of the Spleens 6 and 12 Weeks after Transplantation with Immune Lymphoid Cells

Virus titres of transplanted groups (log values)	6 weeks after transplantation	12 weeks after transplantation
No trace	0/7	4/14
Trace	0 7	1/14
1 2	2/7	7/14
2 3	4/7	1/14
3 4	1 7	1 14
> 4	0 7	0/14
Virus titres of control group		
> 6	10 10	10/10

The Effect of Transplantation of Isologous Lymphoid Cells from Mice Vaccinated with Live Virus

For vaccination of donors the intraperitoneal route was used throughout The maximal dose of LCM virus given by this route which adult AKA mice can survive was found to be 100 times the intracerebral LD 50 dose This amount of virus was therefore chosen as the vaccination dose When normal adult mice receive such an inoculation they develop viraemia, the majority look sick on about the tenth day, and at that time the virus titres of their spleens are at least 10^5 However, all of the inoculated mice will usually recover, and when tested three weeks after the injection only occasionally is a mouse found in which virus is present in the blood or even in the spleen If at that time a new virus dose is given no clinical symptoms occur, and two weeks later none of the mice tested were found to have any detectable virus in blood or spleen

In a pilot experiment spleen and lymph node cells from mice vaccinated twice were transplanted to virus carriers The donor mice received the second vaccination three weeks after the first and the cells were harvested two weeks after the second injection Eight mice were transplanted with these cells and the cell dose was 100×10^6 cells per mouse The virus in the tail blood was titrated four and six weeks later and in the heart blood and spleens nine weeks later The results were

groups were followed for nine weeks and two groups for 12 weeks All groups were titrated on the fifth or the sixth week The mean titres for the individual groups at that time ranged from $10^{0.5}$ to $10^{1.1}$ Figure 3 gives the mean blood titre curve for all five groups of mice As was the case for the first group described, the mean titre curve shows the decline of titre begins one week after the transplantation Between the first and fifth weeks the fall in titre is rather steep, dropping from 10^4 to $< 10^1$, but then the curve begins to level off and from the sixth to the twelfth week only a slight decrease in the mean titres can be demonstrated However, as can be seen in Table 1, from about the sixth week a few mice will be found which show only traces or even no trace of virus in undiluted heart blood Through the ninth to the twelfth week this observation increases in frequency, and by the end of the experiment about 1/3 of the mice have titres which cannot be determined exactly This, of course, influences the mean titre curve, but the degree to which it is influenced is difficult to decide For this reason the curve from the sixth week on is given as a dotted line and this line is drawn among points of mean titre values determined by calculating as zero values those titres which cannot be determined However, even if the curve from the sixth week on is uncertain, it can at least be said that from this time the decline in the blood virus titres is much slower than during the first weeks

TABLE 1

Virus Titres of Heart Blood 6 and 12 Weeks After Transplantion with Immune Lymphoid Cells

Virus titres (log values)	6 weeks after transplantation	12 weeks after transplantation
No trace	1/7	3/14
Trace	1/7	3/14
0 1	4/7	6/14
> 1	1/7	2/14

The numerator gives the number of mice with the virus titre indicated the denominator the number of mice tested

In the experiments described here none of the 46 mice transplanted failed to respond to the transplantation by a great reduction of the virus titre of the blood In the majority the reduction in titre took place within six weeks and in the remaining few before the end of nine weeks Table 1 shows the virus titres of the groups of mice which were titrated 12 weeks after the transplantation It is seen that even so long after the transplantation mice which have titres greater than 10^1 can still be found

The virus titres of the spleens of the virus carriers were also greatly influenced by the transplantation of lymphoid cells from the immune mothers Table 2 shows titres of one of the above-mentioned groups when killed six weeks after the transplantation The spleen titres of

the control groups killed at the same time are also shown in the table. It is clearly apparent that within the first six weeks a very great reduction of the spleen titres occurs. The difference between titres of some mice in the transplanted group and any mouse in the control group was more than 10^4 fold and the mean reduction more than 10^3 fold. After 12 weeks the difference between the two groups is even more pronounced. Four out of the fourteen transplanted mice did not have any detectable virus in the 10 per cent spleen suspension tested. However three of the four mice with no detectable virus belonged to the same group of seven mice and this effect may be unusual.

TABLE 2

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No trace	0/7	4/14
Trace	0/7	1/14
1–2	2/7	7/14
2–3	4/7	1/14
3–4	1/7	1/14
> 4	0/7	0/14
Virus titres of control group		
> 6	10/10	10/10

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For vaccination of donors the intraperitoneal route was used throughout. The maximal dose of LCM virus given by this route which adult AKA mice can survive was found to be 100 times the intracerebral LD_{50} dose. This amount of virus was therefore chosen as the vaccination dose. When normal adult mice receive such an inoculation they develop viraemia, the majority look sick on about the tenth day and at that time the virus titres of their spleens are at least 10^5. However all of the inoculated mice will usually recover, and when tested three weeks after the injection only occasionally is a mouse found in which virus is present in the blood or even in the spleen. If at that time a new virus dose is given no clinical symptoms occur and two weeks later none of the mice tested were found to have any detectable virus in blood or spleen.

In a pilot experiment spleen and lymph node cells from mice vaccinated twice were transplanted to virus carriers. The donor mice received the second vaccination three weeks after the first and the cells were harvested two weeks after the second injection. Eight mice were transplanted with these cells and the cell dose was 100×10^{6} cells per mouse. The virus in the tail blood was titrated four and six weeks later and in the heart blood and spleens nine weeks later. The results were

very similar to those obtained when cells from immune mothers were used. Four weeks after the transplantation all mice except one had blood titres lower than 10^{1}, and the remaining one had a titre of $10^{1.7}$. Six weeks after the transplantation four had titres $< 10^{1}$ and four between 10^{1} and 10^{2}, but nine weeks after all of the blood titres were $< 10^{1}$. The spleen titres at that time were between 10^{1} and 10^{3} in seven mice and one had only traces of virus.

To investigate the influence of a different vaccination status on the immunity which can be conferred by the lymphoid cells from the vaccinated donors, five groups of mice were vaccinated with different numbers of virus inoculations. The first group received four virus inoculations, the second three, the third two and the remaining two only one. The mice in the groups which were given more than one virus injection received the second three weeks after the first and the following inoculations (if any) at intervals of two weeks. The cells were harvested from all these groups two weeks after the last virus inoculation, *i.e.* for one group nine weeks, for the next seven weeks, and for the third five weeks after the first vaccination. Of the two groups which were given only one vaccination the cells from the first were harvested three weeks later, *i.e.* at the end of the infection, at which stage blood and organs were free of virus. The cells from the other group were harvested ten days after the inoculation, *i.e.* at the peak of the infection when blood and organs contained large amounts of virus.

The time-table for the vaccinations was planned in such a way that all groups could be harvested on the same day. The recipients were as usual virus carriers of six to eight weeks of age, chosen by random selection, and one group was kept as control. The lymphoid cells from each of the vaccinated groups were pooled and transplanted to a group of eight recipients in amounts of 100×10 cells per mouse. Four and six weeks later the virus titre of the tail blood was determined and nine weeks later the heart blood and spleens were examined. Figure 4 shows the mean titre curves for the results obtained in each of the groups and Table 3 shows the titrations at the fourth and ninth weeks. It is clearly apparent that four weeks after the transplantation the cells from the mice vaccinated on four, three and two occasions have had the same effect on the virus in the recipients, and for all the three virus carrier groups the mean titres are now below 10^{1}. From the fourth to the sixth week the titres seem to have a tendency to rise and subsequently to decline again slowly. However, as was the case for the mean titre curve for mice receiving cells from immune mothers, these experiments also show that from the sixth week increasing numbers of mice had so little virus in the blood that the titres could not be determined and the points on this part of the curve are therefore very uncertain. The groups of mice which received cells from the donors vaccinated only once also showed a sharp decline in the blood virus titres but this decline did not follow a curve which was quite as steep as the curves in the other

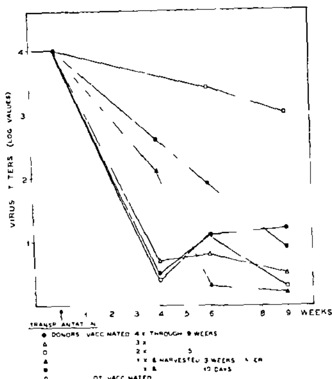


Fig 4

The mean blood virus titre curves for groups of virus carriers transplanted with 100×10^6 lymphoid cells from donors with different states of immunity

TABLF 3

Influence of Vaccination Status of the Donors on the Immunity Conferred

Transplanted groups receiving 100×10^6 cells from donors vaccinated	Virus titres in blood (log values)								Virus titres of spleens (log values)			
	4 weeks after transpl				9 weeks after transpl				9 weeks after transpl			
	1	1 2	2 3	3	< 1	1 2	2 3	3	2	2 3	3-5	5
4 times within 9 weeks	7/8	0 8	1 8	0 8	5 8	2 8	1/8	0 8	6 8	2 8	0 8	0 8
3 times within 7 weeks	6 8	2 8	0 8	0 8	6 8	2 8	0 8	0 8	7 8	1 8	0 8	0 8
Twice within 5 weeks	7 8	1/8	0 8	0 8	8 8	0 8	0 8	0 8	6 8	2 8	0 8	0 8
Once and cells harvested 3 weeks later	1 8	3 8	3 8	1 8	8 8	0 8	0 8	0 8	8 8	0 8	0 8	0 8
Once and cells harvested 10 days later	1 8	0 8	6 8	1 8	5 8	3 8	0 8	0 8	4 8	4 8	0 8	0 8
Controls not transplanted	0 7	0 7	0 7	7 7	0 7	0 7	1/7	6 7	0 7	0 7	0 7	7/7

very similar to those obtained when cells from immune mothers were used. Four weeks after the transplantation all mice except one had blood titres lower than 10^1, and the remaining one had a titre of $10^{1.7}$. Six weeks after the transplantation four had titres $< 10^1$ and four between 10^1 and 10^2, but nine weeks after all of the blood titres were $< 10^1$. The spleen titres at that time were between 10^1 and 10^2 in seven mice and one had only traces of virus.

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TABLE 4

Influence of Cell Dose on Immunity Conferred

Transplanted groups receiving	Virus titres of blood (log values)								Virus titres of spleens (log values)			
	6 weeks after transpl				9 weeks after transpl				9 weeks after transpl			
	1	1 2	2 3	3	1	1 2	2 3	> 3	2	2 3	3-5	-
200 $\times 10^6$ cells	6 8	2/8	0 8	0/8	7 8	1/8	0/8	0 8	4 6	2 6	0 6	0 6
100 $\times 10^6$ cells	12/17	2/17	2/17	1/17	9 16	6/16	1/16	0 16	14/16	2/16	0 16	0/1
50 $\times 10^6$ cells	6/15	4/15	2/15	3/15	10/14	3/14	0 14	1/14	11/14	2/14	1/14	0 1
25 $\times 10^6$ cells	1/17	1/17	2/17	13/17	4/16	3 16	4/16	5 16	3/16	3 16	6/16	4 1
12.5 $\times 10^6$ cells	0 8	0 8	0 8	8 8	0/7	1/7	3/7	3 7	0/7	0 7	4/7	3
Controls not transplanted	0/10	0 10	0/10	10/10	0/10	0/10	0/10	10 10	0/10	0/10	0 10	10 1

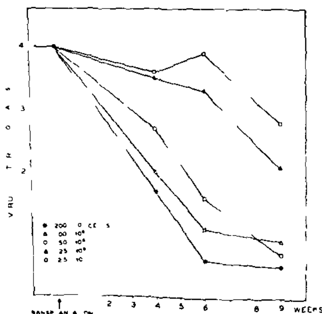


Fig 5

The mean blood virus titre curves for groups of virus carriers transplanted with different amounts of immune lymphoid cells

It has been stated above that the results obtained in the two experiments were very similar. This is true within the first six weeks, but in the ninth week it is true only of the groups of mice receiving at least 50 × 10^6 cells. One of the groups which was given 25 × 10^6 cells shows definitely lower titres in the ninth week than the other, a few of these mice having so little virus in the blood that an exact titre could not be determined. On the other hand, mice having a titre above $10^{3.5}$ could be found in the same group. In all 10 of the mice which received 25 × 10^6

groups For the group transplanted with cells taken from mice only ten days after the virus inoculation this slower decline in titre was especially marked However, nine weeks after the transplantation the mean titres for all groups had reached the same level

Table 3 also shows the titres of the spleens nine weeks after transplantation Among the first four groups no clear difference could be seen and in all of these the majority of the titre values were down to $< 10^2$ The group of mice which had received transplantations from donors vaccinated only ten days before also showed a marked reduction in virus titres but about half of the group still had titres as high as 10^2 to 10^3

The Effect of Cell Dose on the Immunity Conferred

In the preceding experiments a fixed amount of 100×10^6 cells was used throughout In the following, two experiments are described in which varying amounts of cells were transplanted In the first experiment the donors used were mothers of infected babies immunized as described above From the donors the spleen and lymph node cells were pooled and different amounts of these cells transplanted into three groups of virus carriers Eight mice received 100×10^6 cells, nine received 50×10^6 cells, and eight were given 25×10^6 cells In the second experiment the donors were mice vaccinated four times within nine weeks as described above The spleen and lymph node cells from these donors were also pooled and transferred in different amounts to groups of eight to nine virus carriers One group received 200×10^6 cells, the second 100×10^6 cells, the third 50×10^6 cells, the fourth 25×10^6 cells and the fifth 12.5×10^6 cells The recipient groups were as usual selected at random, tested for virus before the experiment and one group was not transplanted and kept as a control In both experiments the virus titres in the tail blood of all mice were titrated individually four and six weeks after the transplantation and in the heart blood and spleens nine weeks after

The results obtained in the two experiments were highly similar and hence are recorded together Some of the titrations are shown in Table 4 and the mean titre curves in Figure 5 It is clearly seen that the mean titre curves for mice receiving 200 and 100×10^6 cells resemble each other greatly, reaching a mean level of $< 10^1$ in six weeks and remaining there The titre curve for the group which received 50×10^6 cells runs a little above the other two in the first six weeks, but at the time of the ninth week it is down to the same level However, the titre curves for the groups of mice receiving either 25 or 12.5×10^6 cells are different For both of these groups the mean titre curves decline very little if at all within the first six weeks and only in the ninth week is it possible to see a fall in titre As expected, this reduction of virus in the blood was most pronounced for the group which received 25×10^6 cells

TABLE 4

Influence of Cell Dose on Immunity Conferred

Transplanted groups receiving	Virus titres of blood (log values)								Virus titres of spleens (log values)			
	6 weeks after transpl				9 weeks after transpl				9 weeks after transpl			
	1	1 2	2 3	3	1	1 2	2 3	> 3	< 2	2 3	3 5	
200 × 10⁶ cells	6/8	2 8	0 8	0 8	7 8	1/8	0 8	0 8	4 6	2 6	0 6	0 6
100 × 10⁶ cells	12/17	2/17	2/17	1/17	9 16	6/16	1/16	0 16	14 16	2 16	0 16	0 1
50 × 10⁶ cells	6 15	4/15	2/15	3/15	10 14	3/14	0 14	1/14	11/14	2 14	1/14	0 1
25 × 10⁶ cells	1/17	1/17	2/17	13 17	4/16	3 16	4/16	5 16	3/16	3 16	6 16	4 1
12.5 × 10⁶ cells	0/8	0 8	0/8	8/8	0/7	1/7	3/7	3 7	0/7	0 7	4/7	3 7
Controls not transplanted	0 10	0 10	0/10	10 10	0/10	0 10	0/10	10 10	0/10	0 10	0 10	10 1

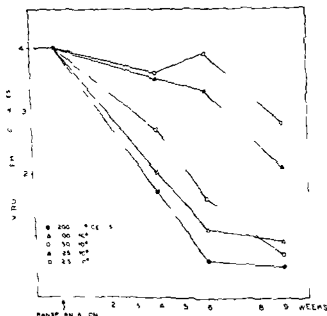


Fig 5

The mean blood virus titre curves for groups of virus carriers transplanted with different amounts of immune lymphoid cells

It has been stated above that the results obtained in the two experiments were very similar. This is true within the first six weeks, but in the ninth week it is *true only of the* groups of mice receiving at least 50 × 10⁶ cells. One of the groups which was given 25 × 10⁶ cells shows definitely lower titres in the ninth week than the other, a few of these mice having so little virus in the blood that an exact titre could not be determined. On the other *hand*, mice having a titre above $10^{3.5}$ could be found in the same group. In all, 10 of the mice which received 25 × 10⁶

groups For the group transplanted with cells taken from mice only ten days after the virus inoculation this slower decline in titre was especially marked However, nine weeks after the transplantation the mean titres for all groups had reached the same level

Table 3 also shows the titres of the spleens nine weeks after transplantation Among the first four groups no clear difference could be seen and in all of these the majority of the titre values were down to $< 10^{\cdot}$ The group of mice which had received transplantations from donors vaccinated only ten days before also showed a marked reduction in virus titres but about half of the group still had titres as high as 10^2 to 10^3

The Effect of Cell Dose on the Immunity Conferred

In the preceding experiments a fixed amount of 100×10^6 cells was used throughout In the following, two experiments are described in which varying amounts of cells were transplanted In the first experiment the donors used were mothers of infected babies immunized as described above From the donors the spleen and lymph node cells were pooled and different amounts of these cells transplanted into three groups of virus carriers Eight mice received 100×10^6 cells, nine received 50×10^6 cells, and eight were given 25×10^6 cells In the second experiment the donors were mice vaccinated four times within nine weeks as described above The spleen and lymph node cells from these donors were also pooled and transferred in different amounts to groups of eight to nine virus carriers One group received 200×10^6 cells, the second 100×10^6 cells, the third 50×10^6 cells, the fourth 25×10 cells and the fifth 12.5×10^6 cells The recipient groups were as usual selected at random, tested for virus before the experiment and one group was not transplanted and kept as a control In both experiments the virus titres in the tail blood of all mice were titrated individually four and six weeks after the transplantation and in the heart blood and spleens nine weeks after

The results obtained in the two experiments were highly similar and hence are recorded together Some of the titrations are shown in Table 4 and the mean titre curves in Figure 5 It is clearly seen that the mean titre curves for mice receiving 200 and 100×10 cells resemble each other greatly, reaching a mean level of $< 10^1$ in six weeks and remaining there The titre curve for the group which received 50×10^6 cells runs a little above the other two in the first six weeks, but at the time of the ninth week it is down to the same level However the titre curves for the groups of mice receiving either 25 or 12.5×10^6 cells are different For both of these groups the mean titre curves decline very little if at all within the first six weeks and only in the ninth week is it possible to see a fall in titre As expected, this reduction of virus in the blood was most pronounced for the group which received 25×10 cells

TABLE 5

Ability of Normal Lymphoid Cells to Confer Immunity

Transplanted groups receiving 100 × 10^6 normal cells	Virus titres of blood (log values)								Virus titres of spleens (log values)		
	6 weeks after transpl				9 weeks after transpl				9 weeks after transpl		
	< 1	1 2	2-3	> 3	1	1 2	2-3	3	3-4	4-5	> 5
1	0 8	0 8	1/8	7 8	0 8	0 8	0/8	8 8	0 8	1/8	7/8
2	0 8	2 8	2 8	4 8	0/8	1/8	3/8	4 8	1/8	3 8	4/8
3	0 8	1 8	1/8	6 8	0 8	1/8	3/8	4 8	1/8	2 8	5/8
4	0 8	0 8	1/8	7/8	0/8	2/8	1/8	5 8	1/8	1/8	6/8
Controls not transplanted	0 7	0 7	0/7	7/7	0/7	0/7	0/7	7/7	0/7	0 7	7/7

When the spleens were titrated only 10 had significantly reduced titres i e below 10^5 Of these the lowest titre measured was 10^3 and the majority were between 10^4 and 10^5

To summarize normal cells in the amounts given seem to be able to confer immunity but the effect obtained is late and only in about 25 per cent of the recipients a significant reduction of the virus titres in blood and spleen has occurred nine weeks after the transplantation

The Inability of Killed Cells Homologous Cells and Immune Plasma to Confer Immunity

The investigators working on transplantation immunity problems all agree that if immunity to skin and tumor grafts is to be transferred, and if a tolerant state to such grafts is to be terminated only live immunologically competent cells from the same highly inbred strain of animals can be used Injections of killed cells homologous cells or immune plasma cannot confer any immunity or bring a tolerant state to an end In order to see whether this is also true of the immunity conferred to virus carriers the following experiments were carried out

The effect of killed immune isologous cells The spleen and lymph node cells from a group of immunized AKA mothers of infected babies were pooled and then divided into equal parts each containing 100 × 10^6 cells per 0 5 ml One of the suspensions was left intact whilst the other was quickly frozen to minus 60° C kept frozen for about two minutes thawed under running tap water and then carefully shaken to break up the clumps The two suspensions were given to different groups of virus carriers in doses of 0 5 ml intravenously The injection of unfrozen cells was as usual a harmless affair However when the cells are killed by freezing and thawing they are extremely dangerous to inject intravenously If given quickly the recipients will die immediately but if the injections are carried out very slowly about half of the mice can survive In our experiment nine mice survived the injection of killed cells Three weeks after the transplantation the virus

cells had titres which showed that a significant reduction had occurred, *i e* they had titres lower than 10^{6} Of the seven mice which received $12\ 5 \times 10^{6}$ cells the lowest titre measured was $10^{1\ 4}$, most titres were between 10^{2} and 10^{3} and three mice had titres higher than $10^{3\ 5}$ In all, only three of the mice had titres which showed that a significant reduction had occurred

The spleen titres in the groups of mice receiving from 200 to 50×10^{6} cells all showed the same pattern Most titres were between 10^{1} and 10^{2}, a few had only traces of virus left and one had as much as 10^{3} As regards the 17 mice which received 25×10^{6} cells, only three had titres lower than 10^{2} but titres as high as $10^{3\ 5}$ were also found As was the case in the heart blood, the spleens in one of the two groups had definitely lower titres than the other The highest titre seen in this group was $10^{3\ 6}$ Among the seven mice which received $12\ 5 \times 10^{6}$ cells the lowest spleen titre found was $10^{1\ 8}$ Only four mice had titres which showed that a significant reduction had occurred, *i e* titres below 10^{2}, and three mice had titres higher than $10^{3\ 5}$

On the whole it can be said that for mice receiving from 200 to 50×10^{6} cells the mean titre curves follow the same slope However, even 25 and $12\ 5 \times 10^{6}$ cells have some effect on the virus titres of transplanted virus carriers but the effect is late, often weak, and characterized by great individual variation

Ability of Normal Cells to Confer Immunity

Billingham, Brent & Medawar (1) have shown that a tolerant state to a skin graft can also be terminated by injecting normal lymphoid cells Experiments with the purpose of investigating the effect of normal lymphoid cells on the virus carrier state were therefore carried out As a cell dose of 100×10^{6} immune cells had been found to be well above the dose necessary to give satisfactory effect, the normal cells were also transplanted in amounts of 100×10^{6} per mouse Four experiments were carried out using a total of 32 recipients In each of the experiments the spleen and lymph node cells from a group of normal adult AKA mice were harvested, pooled and injected intravenously into groups of virus carriers Six weeks later the virus titres of the tail blood were determined individually for each mouse and nine weeks later the heart blood and spleens were titrated The mean titre curve for all the 32 recipient mice is shown as the upper curve in Figure 4 and the results obtained in each group are given in Table 5 It is apparent that six weeks after the transplantation only three out of 32 transplanted mice had titres which were $< 10^{2}$ and 24 had titres $> 10^{3}$ Nine weeks later four out of the 32 *mice* had blood titres which were $< 10^{2}$ but 21 still had titres $> 10^{3}$ Four of the titres between 10^{2} and 10^{3} were below $10^{2\ 6}$ and therefore at the end of the experiment a total of eight mice had titres which can be considered significantly reduced

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Transplanted groups receiving 100 × 10⁶ normal cells	Virus titres of blood (log values)								Virus titres of spleens (log values)		
	6 weeks after transpl				9 weeks after transpl				9 weeks after transpl		
	<1	1-2	2-3	>3	1	1-2	2-3	3	3-4	4-5	>5
1	0 8	0 8	1/8	7/8	0/8	0/8	0/8	8 8	0/8	1 8	7/8
2	0 8	2 8	2 8	4 8	0 8	1/8	3/8	4 8	1/8	3 8	4 8
3	0 8	1/8	1/8	6 8	0 8	1/8	3/8	4 8	1 8	2 8	5 8
4	0 8	0 8	1/8	7/8	0 8	2/8	1/8	5 8	1/8	1/8	6/8
Controls not transplanted	0 7	0 7	0 7	7 7	0/7	0/7	0 7	7/7	0/7	0 7	7/7

When the spleens were titrated only 10 had significantly reduced titres i e below 10^5 Of these the lowest titre measured was 10^3 and the majority were between 10^4 and 10

To summarize normal cells in the amounts given seem to be able to confer immunity but the effect obtained is late and only in about 25 per cent of the recipients a significant reduction of the virus titres in blood and spleen has occurred nine weeks after the transplantation

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TABLE 6

Inability of Killed Cells Homologous Cells and Immune Plasma to Confer Immunity

Transplanted groups receiving	Virus titres of blood (log values) 1 week after transpl 1	1 2	2 3	3	3 weeks after transpl 1	1 2	2 3	> 3	6 weeks after transpl 1	1 2	2 3	> 3	Virus titres of spleens (log values) 6 weeks after transpl < 2	2 3	3-5	> 5
Live immune isologous lymphoid cells	0/10	0/10	1/10	9/10	1/10	6/10	1/10	2/10	8/10	1/10	1/10	0/10	6/10	3/10	1/10	0/10
Killed immune isologous lymphoid cells					0/9	0/9	0/9	9/9	0/9	0/9	1/9	8/9	0/9	0/9	0/9	9/9
Live immune homologous lymphoid cells	0/7	0/7	0/7	7/7	0/7	0/7	0/7	7/7	0/13	0/13	3/13	10/13	0/13	0/13	0/13	13/13
Immune isologous plasma	0/7	0/7	1/7	6/7	0/7	0/7	0/7	7/7	0/12	0/12	3/12	9/12	0/12	0/12	0/12	12/12
Controls not transplanted									0/8	0/8	2/8	6/8	0/8	0/8	0/8	8/8

titres of the tail blood were determined and six weeks after transplantation all mice were killed and the virus titres of blood and spleens determined The results are shown in Table 6 The mice which received the live cells all showed a clear cut response to the transplantation the blood and spleen titres being reduced to the expected level However none of the mice which received the killed cells showed a significant response One mouse had a blood titre of $10^{\cdot 9}$ but all the remaining titres were more than 10^{3} All spleen titres were higher than $10^{\ 5}$

The effect of homologous immune lymphoid cells Two experiments were carried out in which homologous immune lymphoid cells were transplanted to virus carriers In the first experiment C_3H mice were used as donors to AKA carriers The C_3H donor mice were mothers of babies infected at birth and two weeks before harvesting the lymphoid cells these mice were given 100 LD 50 doses of live LCM virus intraperitoneally *i e* they were treated in exactly the same way as the immunized AKA mothers which in the above experiments were found to provide immune cells with a high activity In the second experiment the donors used were the institute s ordinary stock of white mice, immunized by two intraperitoneal injections of 100 I D 50 doses of live virus The two virus inoculations were given three weeks apart and the cells harvested two weeks after the second vaccination dose, *i e* the immune status of these cells should closely correspond to that of the cells taken from twice vaccinated AKA donors which in the foregoing experiments were found to be very active cells

Spleen and lymph node cells were harvested and pooled separately for each of these two donor groups From the C_3H cell pool six and from the other seven AKA virus carriers were transplanted intravenously with 100×10^{6} cells per mouse One week and three weeks after the transplantation the second group of recipients was tested for virus in the tail blood Six weeks after the transplantation all the transplanted mice were killed and the virus titres of the heart blood and spleens determined individually in each mouse The results for the two groups were highly similar and hence are recorded together in Table 6 It is clearly seen that no detectable decline in titre had taken place within the first three weeks After six weeks three out of 13 mice had titres below 10^{3} but all had titres higher than $10^{\ [illegible]}$ *i e* none had titres which could be considered significantly reduced That no decline in titre had in fact been caused by the transplantation is confirmed by the finding that none of the spleen titres showed any significant reduction all titres being higher than $10^{5\ 5}$ Moreover in the untreated controls two out of eight mice showed blood virus titres as low as those found in the transplanted groups

The effect of immune isologous plasma Two experiments were performed in which plasma from immune AKA mice was injected into virus carriers In the first experiment immunized mothers of infected AKA babies were used as donors and in the second twice vaccinated

AKA mice were the sources of immune plasma In both experiments the mice were bled from the heart, the blood was pooled and just enough heparin was added to prevent clotting After centrifugation the plasma was collected From the plasma pool from immunized mothers five virus carriers were given undiluted plasma intravenously in amounts of 0 5 ml per mouse From the plasma pool from the twice vaccinated mice seven virus carriers received the same volume of undiluted plasma intravenously One week and three weeks after the injection the virus titres of the tail blood in the second group were determined and six weeks after the injection all mice were killed and the virus in the blood and spleens titrated The results obtained in the two groups were highly similar and hence are recorded together in Table 6 It can be seen that the immune plasma had no detectable effect on the virus, either shortly after it was given or later After six weeks three out of the 12 injected mice had titres lower than 10^3 However, analogously with carriers transplanted with homologous cells, none of the recipients in this experiment showed titres below $10^{2.5}$, i e below the limit for "normal" titres or below the titres found in the controls Moreover, as all spleen titres were found to be higher than $10^{3.5}$, it can be concluded that no significant reduction of the virus titres had been caused by the immune plasma within the observation period of six weeks

As an extra control of the immunity in the two groups of donors used in each experiment the lymphoid cells of the donors were also harvested and transplanted to virus carriers in doses of 100 × 10^6 cells per mouse In both cases the usual decline in virus titres was caused by the cell transplantation

TABLE 7

Influence of Sex Differences Between Donors and Recipients on Immunity Conferred

Sex of donors	Sex of recipients	Virus titres of blood (log values) 6 weeks after transpl				Virus titres of spleens (log values) 6 weeks after transpl			
		1	1 2	2 3	3	2	2 3	3-5	5
female	female	5/7	2/7	0/7	0 7	5 7	2/7	0/7	0 7
female	male	6/8	1/8	1/8	0 8	3/8	5/8	0/8	0 8
male	male	5/7	1/7	1/7	0 7	6/7	1/7	0/7	0 7
male	female	5/7	2/7	0 7	0/7	5/7	1/7	1/7	0 7

The Influence of Sex Difference between Donors and Recipients on the Immunity Conferred

As it was found that cells from homologous donors do not confer any immunity, and as there is strong support for the assumption that the Y chromosome carries its own histocompatible genes (4, 6), experiments were carried out to determine the effect of sex differences on the

immunity conferred Cells were harvested from immune donors and injected both into male and female virus carriers, in doses of 100×10^6 cells per mouse The effect on the virus titres of blood and spleens was tested six weeks later The experiment and the results are shown in Table 7 From this it can be seen that sex differences between donors and recipients has no apparent influence on the immunity conferred

The Influence of Transplantation Route on the Immunity Conferred

In two experiments spleen and lymphoid cells from the same batch were given intravenously to one group and intraperitoneally to another, in amounts of 100×10^6 cells per inoculation When tested later it was not possible to detect any clear cut difference in the immunity conferred Perhaps cells given intravenously caused a more rapid and steep fall in the blood virus titres, but the differences seen are hard to evaluate in view of the individual variations which always occur

Inability of Immune Lymphoid Cells to Influence the Course of an Acute Infection

As the transplantation of immune lymphoid cells had such a pronounced effect on inapparent infections, experiments were carried out to see whether an effect on acute infections could be demonstrated Mothers of infected babies immunized with live virus two weeks before the cells were harvested, as described above, were used as source of immune cells Each batch of cell suspension was tested for virus In no case could any trace of virus be demonstrated

TABLE 8
Inability of Immune Lymphoid Cell Transplantations to Influence the Course of Acute Infections in Normal Mice

Groups transplanted	Transpl route No of cells given	Infection route Virus dose	No of mice surviving
3 weeks before infection	100×10^6 i v	100 LD 50 i c	10/10
2 weeks before infection	100×10^6 i v	100 LD 50 i c	8/10
1 week before infection	100×10^6 i v	100 LD 50 i c	0/10
3 days before infection	100×10^6 i v	100 LD 50 i c	0/10
On infection day	100×10^6 i v	100 LD 50 i c	0/10
2 days after infection	100×10^6 i v	100 LD 50 i c	0/10
4 days after infection	100×10^6 i v	100 LD 50 i c	0/10
6 days after infection	100×10^6 i v	100 LD 50 i c	0/10
Control	none	100 LD 50 i c	0/10

Eight groups of normal adult AKA mice received by intravenous inoculations spleen and lymphoid cells from these donors, in doses of 100×10^6 cells per mouse At different times in relation to the transplantation the mice were challenged with 100 LD 50 doses of virus

intracerebrally One group received the virus challenge three weeks after the transplantation, one two weeks after, one one week after, one three days after and one on the day of transplantation, within one hour of the cells being given The other three groups were transplanted two to six days after the virus challenge and finally one group was kept as a control and received the virus inoculation only The time-table was arranged in such a way that the virus challenge was given to all mice on the same day The results are shown in Table 8 It is clearly apparent that only transplantations given as early as two weeks before the virus challenge had any protective effect In none of the other groups could protection be demonstrated

The whole experiment was repeated exactly as described but with the one difference that the doses of challenging virus were reduced to 10 LD 50 The results were as in the foregoing experiment except that with this small virus dose the protective effect could also be demonstrated when the transplantation was given one week before the virus challenge However, no transplantation given later than this had any effect on the course of the infections

DISCUSSION

In the preliminary report it was shown that the LCM virus carrier state in mice can be greatly influenced by adoptive immunization, just as a tolerant state to skin and tumor grafts can be influenced by the same kind of procedure The experiments described here confirm this observation and show other points of resemblance between the results obtained in tissue transplantation immunity studies and in the studies of immunity in virus carriers As in the tissue transplantation experiments (1, 2, 12, 13), it has been possible only to confer immunity to the virus in virus carriers by means of live, immune isologous lymphoid cells, and if killed immune cells, homologous immune cells or immune plasma are used no effect has been demonstrated during an observation period of six weeks[1] Moreover, the cell dose necessary to cause a satisfactory response in virus carriers is of the same order as the dose necessary for conferring an immunity to tumor grafts, i e about 50×10^6 immune lymphoid cells Furthermore, just as *Billingham, Brent & Medawar* (1) found that normal lymphoid cells can confer an immunity to immunologically tolerant animals, the data presented here show that transplantation of normal lymphoid cells can influence the virus carrier state However, as in the tissue transplantation experiments, it was found that the effect of normal cells on virus carriers is much weaker and takes much longer to develop than if immune cells are transplanted All these results give further support for the assumption that the LCM

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virus carrier state in mice may be due to the phenomenon of immunological tolerance. It is true that the investigators working with tolerance to skin and tumor grafts talk about terminating the tolerant state, whereas the present author has not yet succeeded in completely eliminating the virus and cannot therefore claim that the tolerant state to the virus is brought to an end. However, this difference is perhaps only apparent. When working with grafts it may be very difficult to exclude the possibility of a persistence of some tolerance, whereas virus titrations are perhaps more sensitive.

The many points of resemblance between the results of the tissue transplantation immunity studies and those obtained with virus carriers offer a temptation to assume that the immunity to the virus conferred on virus carriers is similar in type to the immunity to skin and tumor grafts which can also be conferred. The mechanism of this immunity has been discussed in detail by others (5, 9, 10) and hence this argumentation will not be repeated here. However, it can be concluded, as stated by *Medawar* (9), that all parties to tissue transplantation immunity experiments seem to agree that the immunity is transferred by what is in effect a repopulation or cellular grafting of the secondary host with living, immunologically activated cells from the primary host. However, the minds of all are also open to the possibility of a sympathetic activation or transduction of the recipient's own lymphoid cells by subcellular ingredients from the donor.

The question whether the experience gained with the LCM virus in mice can be of any use when dealing with other virus infections remains to be answered. However, according to *Rubin* (19) and *Gross* (7) avian leukosis virus and mouse leukemia virus seem to be able to cause a tolerant virus carrier state similar to that seen with the LCM virus. Moreover, when more is known about other tumor viruses, and even about the ordinary viruses which admittedly can cause latent infections, it may be discovered that a tolerant state to a virus is not as unusual as we are inclined to think today. To know the immune mechanism of such infections and how to influence the carrier state could be of interest.

As the other viruses at present are rather difficult to work with, the LCM virus may be considered as the best working model. However, if inbred strains of chickens are available, avian leukosis virus may be even better, and in addition may give direct information about the possibility of preventing the development of tumors in the virus carriers.

In the experiments presented here it has been shown that by titrating the virus in the blood and spleens the response to a transplantation of lymphoid cells can be followed quite accurately. Information concerning the influence of different transplantation factors has thereby been obtained.

With the kind of immune cells and the doses used in the experiments

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dose Moreover when a donor has been exposed to the virus only once the lymphoid cells are able to confer immunity for a long time Very active cells can be harvested two to three weeks after virus inoculations and in experiments not recorded here donors which have not been in contact with any virus for 6 weeks or more have been used and the cells from these mice were as effective as any These findings are different from those in immunity experiments with tumor grafts In the latter to obtain a good result the lymphoid cells have to be harvested within a very narrow time interval at a special time after the donors have been exposed to the antigen (13) It seems probable that also this difference between the two kinds of experiments is due to the difference in antigens The virus antigen can multiply and persist for a long time in the animal thereby causing a long lasting strong stimulus to immunity whereas grafts of skin and tumors do not multiply for very long if at all and will quickly be disposed of by the hosts The stimulus to immunity produced by such antigens must be much weaker and of much shorter duration

As indicated in the discussion above it seems possible to give a reasonable explanation of the differences observed between immunity experiments with skin and tumor grafts and those where a virus is concerned The existence of these differences therefore does not speak against the assumption that the mechanisms involved in the two types of immunity are similar to one another Nevertheless so little is known about this kind of immunity that our minds have to be open to other possibilities First of all it must be strongly emphasized that the only indication of an immunity conferred to virus carriers is the observation of a decline in virus titre A specific or perhaps even an unspecific destruction of the virus producing cell system may be the only reason for this virus reduction and immunity phenomena may not have anything to do with it Other explanations can perhaps also be offered However for the time being the immunity theory seems to be the only one which has experimental support

The transplantation of virus carriers with immune lymphoid cells has usually been a harmless procedure and the decline in virus titres has not been associated with any signs of disease Therefore the adoptive immunization experiments reported here do not support the assumption of *Rowe* (15) and *Hotchin* (8) that an immunological response to the LCM virus should cause a clinical illness However now and then on very rare occasions the transplantations have had a harmful effect on the virus carriers When this happened it has been associated with certain batches of cells and occurred in such a way that all mice receiving cells from these batches became ill and some died about three weeks after the transplantation Moreover if a batch of cells caused clinical disease then the response to the virus was found to be slower and weaker than normal Microscopic examinations of organs from mice which died from the transplantations showed no inflamma

described here it is found first of all that a time interval of at least one week has to elapse before a demonstrable decline in virus titre can be observed This is contrary to what is seen when immunity to skin and tumor grafts is conferred by adoptive immunization (1, 2, 3, 12, 13) In all those cases the effect of the transplantation is demonstrable within a few days Perhaps the difference in reaction time is only apparent, although the fact that transplantations of immune lymphoid cells to normal mice as early as three days before a virus challenge fails to alter the course of the infection also seems to indicate that the transplantation response to LCM virus is a late reaction

The fact that mice were protected by transplantations of immune lymphoid cells given earlier than three days before the virus challenge does not, however, necessarily mean that it is a transplantation effect which is seen Even if no detectable virus was found in the transplanted cells, there could nevertheless have been undetectable amounts of virus present which might have caused an active immunization of the recipients Such an immunity would begin to appear one week after the inoculation, *i e* at the time protection is found, but also at the time when the adoptive immunization could begin to have an effect

The increasing transplantation effect on the virus in the course of at least four to six weeks is an interesting observation In all other transplantation experiments reported, the immunity conferred by adoptive immunization rapidly decreases and can even completely disappear during the same period of time The difference between our experimental conditions and those of others is that in the virus carriers the virus, and thereby the antigenic stimulus, is present for a long time, whereas this is not the case in most other transplantation experiments This constant antigenic stimulus could be the reason for the long lasting and even increasing transplantation effect in virus carriers

The observation that the steep fall in virus titres did not cause a complete elimination of the virus but ended in a stabilized low level of virus came as a surprise Perhaps higher cell doses or repeated transplantations could have depressed the virus completely Perhaps the observation period has not been long enough to show that this would eventually have occurred even under our experimental conditions This remains to be seen However, we already know from a preliminary experiment that if lymphoid cells from transplanted virus carriers nine weeks after the transplantation are transferred to fresh virus carriers, a definite depression of the titres will occur in the new hosts Therefore, these cells have not lost their "power" to take care of even large amounts of virus Why they do not eliminate the minute amount of virus left in the first hosts is open to discussion

Experiments in which cells were transplanted from donors vaccinated in different ways showed that even cells from the most highly immunized mice did not depress the virus titres of the recipients to a level lower than could be obtained with cells from mice given only one vaccination

dose Moreover when a donor has been exposed to the virus only once the lymphoid cells are able to confer immunity for a long time Very active cells can be harvested two to three weeks after virus inoculations and in experiments not recorded here donors which have not been in contact with any virus for 6 weeks or more have been used and the cells from these mice were as effective as any These findings are different from those in immunity experiments with tumor grafts In the latter to obtain a good result the lymphoid cells have to be harvested within a very narrow time interval at a special time after the donors have been exposed to the antigen (13) It seems probable that also this difference between the two kinds of experiments is due to the difference in antigens The virus antigen can multiply and persist for a long time in the animal thereby causing a long lasting strong stimulus to immunity whereas grafts of skin and tumors do not multiply for very long if at all and will quickly be disposed of by the hosts The stimulus to immunity produced by such antigens must be much weaker and of much shorter duration

As indicated in the discussion above it seems possible to give a reasonable explanation of the differences observed between immunity experiments with skin and tumor grafts and those where a virus is concerned The existence of these differences therefore does not speak against the assumption that the mechanisms involved in the two types of immunity are similar to one another Nevertheless so little is known about this kind of immunity that our minds have to be open to other possibilities First of all it must be strongly emphasized that the only indication of an immunity conferred to virus carriers is the observation of a decline in virus titre A specific or perhaps even an unspecific destruction of the virus producing cell system may be the only reason for this virus reduction and immunity phenomena may not have anything to do with it Other explanations can perhaps also be offered However for the time being the immunity theory seems to be the only one which has experimental support

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SUMMARY

Transplantation of isologous immune lymphoid cells to LCM virus carriers causes a marked decrease in the virus titres in blood and spleen.

The transplantation effect is first demonstrable one to two weeks after the cells are transplanted, and reaches its maximum five to six weeks later.

A total elimination of the virus in the carriers has not been achieved. When the virus titre is brought down to just about the detectable level it seems to stabilize and if further reduction occurs it is very slow.

Cell doses of between 50×10^6 and 200×10^6 immune lymphoid cells cause the same reduction in virus titre. Smaller doses also influence the virus but the effect is slow, weak and irregular.

Transplantations of lymphoid cells from donors vaccinated only once give as good results as those obtained with cells from donors vaccinated repeatedly.

Lymphoid cells with a good immunity can be harvested from ten days to at least three weeks after the mice have been in contact with the virus.

Normal isologous lymphoid cells can transfer immunity to virus carriers but the effect of such cells is weak and equivocal.

The intraperitoneal and intravenous transplantation routes seem to be equally effective.

Sex differences between donors and recipients do not influence the transplantation results.

Transplantations of killed immune isologous lymphoid cells, homologous immune lymphoid cells or injections of immune isologous plasma have not caused any effect on the virus during an observation period of six weeks.

Immune lymphoid cells must be transplanted to normal mice at least one week before a virus challenge is made in order to give any protection.

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REFERENCES

1 *Billingham R E, Brent L & Medawar P B* Quantitative Studies on Tissue Transplantation Immunity 2 The Origin, Strength and Duration of Actively and Adoptively Acquired Immunity Proc Roy Soc London B *143* 58 1954

2 *Billingham R F Brent L & Medawar P B* Quantitative Studies on Tissue Transplantation Immunity 3 Active Aquired Tolerance Phil Trans Roy Soc London *s B 239* 357 1955–56

3 *Billingham R F & Brent L* Quantitative Studies on Tissue Transplantation Immunity 4 Introduction of Tolerance in New Born Mice Phil Trans Roy Soc London *s B 242* 439 1958 60

4 *Billingham R E & Silvers W K* Studies on Tolerance of the Y Cromosome

5

6

7

New York 1961

8 *Hotchin J & Weigand H* Studies on Lymphocytic Choriomeningitis in Mice J Immunol *86* 392 1961

9 *Medawar P B* Reaction to Homologous Tissue Antigens in Relation to Hypersensitivity In Cellular and Humoral Aspects of the Hypersensitive States H S Lawrence ed P B Hoeber Inc New York 1959

10 *Medawar P B* *Immunological Tolerance Nature 189 No 4758 14 1961*

11 *Mitchison N A* Passive Transfer of Transplantation Immunity Nature *171* 267 1953

12 *Mitchison N A* Passive Transfer of Transplantation Immunity Proc Roy Soc London *s B 142* 72 1954

13 *Mitchison N A* Studies on the Immunological Response to Foreign Tumor Transplants in the Mouse 1 The Role of Lymph Node Cells in Conferring Immunity by Adoptive Transfer J Exp Med *102* 157 1955

14 *Reed L J & Muench H* Simple Method of Estimating 50 per cent Endpoints Am J Hyg 27 493 1938

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4 *Billingham R F & Silvers W K* Studies on Tolerance of the Y Cromosome

5

6

7 *Cross L* Mouse Leukemia In Oncogenic Viruses L Gross ed Pergamon Press New York 1961

8 *Hotchin J & Weigand H* Studies on Lymphocytic Choriomeningitis in Mice J Immunol 86 392 1961

9 *Medawar P B* Reaction to Homologous Tissue Antigens in Relation to Hypersensitivity In Cellular and Humoral Aspects of the Hypersensitive States H S Lawrence ed P B Hoeber Inc New York 1959

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14 *Reed L J & Muench H* Simple Method of Fstimating 50 per cent Endpoints Am J Hyg 27 493 1938

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3 *Billingham R E & Brent L* Quantitative Studies on Tissue Transplantation Immunity 4 Introduction of Tolerance in New Born Mice Phil Trans Roy Soc London *s B* 242 439 1958–60
4 *Billingham R F & Silvers W K* Studies on Tolerance of the Y Cromosome Antigen in Mice J Immunol 85 14 1960
5 *Chase M W* Immunological Tolerance Ann Rev Microbiol *13* 349 1959
6 *Eichwald F J Silmser C R & Wheeler N* The Genetics of Skin Grafting Ann New York Acad Sci *64* 737 1957
7 *Gross L* Mouse Leukemia In Oncogenic Viruses L Gross ed Pergamon Press New York 1961
8 *Hotchin J & Weigand H* Studies on Lymphocytic Choriomeningitis in Mice J Immunol 86 392 1961
9 *Medawar P B* Reaction to Homologous Tissue Antigens in Relation to Hyper sensitivity In Cellular and Humoral Aspects of the Hypersensitive States H S Lawrence ed P B Hoeber Inc New York 1959
10 *Medaware P B* Immunological Tolerance Nature *189* No 4758 14 1961
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14 *Reed L J & Muench H* Simple Method of Estimating 50 per cent Endpoints Am J Hyg 27 493 1938
15 *Rowe W P* Studies on Pathogenesis and Immunity in LCM Virus Infection in the Mouse U S Naval Med Research Inst Bethesda Md Prog NM 005 0481407 Research Rep *12* 167 1954
16 *Rubin H Fanshier L Cornelius A & Hughes W F* Tolerance and Immunity in Chickens After Congenital and Contact Infections with Avian Leukosis Virus Virology *17* 143 1962
17 *Volkert M* Studies on Immunological Tolerance to LCM Virus A Preliminary Report on Adoptive Immunization of Virus Carrier Mice Acta path et microbiol scandinav *56* 305 1962

FROM THE VIRUS DEPARTMENT, NATIONAL INSTITUTE OF PUBLIC HEALTH, OSLO, NORWAY (HEAD: O LAHELLE, M D)

THE NORMAL ALLANTOIC ANTIGEN WHICH NEUTRALIZES THE INFLUENZA VIRUS HI-ANTIBODY TO HOST MATERIAL

By

ARILD HARBOE

Received 6 xii 62

In the preceding paper the characteristics of the haemagglutination inhibiting (HI) antibodies against the host material of egg-grown myxoviruses were examined (3) The present paper is concerned with the serologic and chemical properties of the normal allantoic antigen which corresponds to this host specific HI-antibody

MATERIALS AND METHODS

Viruses, immune sera and HI-test The influenza A strain PR 8 and B-strain Lee were employed. The sera were obtained from intravenously injected rabbits, and were pretreated with cholera filtrate (Philips-Duphar) The virus antigens in the HI-tests had been purified by means of adsorption to and elution from fowl red cells When, as occasionally happened, the virus stuck to the red cells instead of eluting, one volume of cholera filtrate was added to four volumes of the saline The HI-tests took place in plastic plates at room temperature For further details see the preceding paper (3)

Haemagglutination inhibition neutralizing (HIN) titre The HIN titre of a host antigen was defined as the reciprocal value of the antigen dilution which just neutralized the inhibitory activity of a certain amount of host specific antibody in a modified HI-test against a certain number of haemagglutinating virus doses Before the HIN-titre of a normal allantoic fluid was determined the fluid was treated with an equal volume of cholera filtrate over night at 37° C followed by heating for one hour at 56° C and absorption for ½ hour at 0° C with 10 per cent packed fowl red cells —To serial 2-fold dilutions of the pretreated allantoic fluid was added the suitably diluted, cholera filtrate treated immune rabbit serum and ½ hour later a 0 5 per cent suspension of fowl red cells and immediately afterwards the virus eluate, be [illegible] added between 3 and 6 [illegible] this serum dilution had [illegible]ed in volumes of 0 25 ml [illegible]een purified from crude cholera filtrate by means of two cycles of adsorption to fowl red cells and elution into acetate saline to which had been added 0 1 per cent of $CaCl_2$ The acetate saline consisted of one volume of 0 2 N acetate buffer pH 6 2 and four volumes of normal saline The titre of the enzyme preparation was 640 with PR 8 as a test virus (1)

Bovine pancreatic trypsin Trypure "NOVO" a crystalline preparation which contained 25 Anson units per g was employed

Bacterial trypsin The enzyme had been prepared from a *Bacillus subtilis* culture It was crystalline and contained 27 5 Anson units per g Optimal temperature was 50–60° C.

α-Chymotrypsin The enzyme was crystalline, and the strength was 570 rennet units per g

The latter three preparations were kindly placed at the author's disposal by "NOVO" Pharmaceutical Company, Copenhagen

Papain and ficin were crude commercial preparations

RESULTS

1 *The Specificity of the HIN Antigen in the Allantoic Fluids*

In the following experiment 0 25 ml of cholera treated, normal allantoic fluid, and in a parallel titration normal saline instead of the fluid, was added to each of the serum dilutions in the plates and left to react for ½ hour before the red cells and the virus antigen were added as in a usual HI test The same normal chick, turkey and duck allantoic fluids were employed

An anti chick allantoic PR-8 serum gave the titre 1920 against chick allantoic Lee The titre in the presence of the homologous (chick) allantoic fluid was < 12, with turkey fluid 288, with duck fluid 144 An anti turkey allantoic PR 8 serum gave the titre 384 with turkey allantoic Lee The titre in the presence of the homologous (turkey) allantoic fluid was < 12, with chick fluid 384, with duck fluid 24 The HI neutralization by the heterologous, normal allantoic fluids demonstrates the existence of an antigenic relationship between them However, the more pronounced neutralization by the homologous fluids shows that there is also an antigenic difference between them

Attempts to neutralize the HI-antibody in an antiserum against normal chick allantoic fluid by treating the serum with suspensions of viscera from chick embryo, mouse lung unheated or boiled guinea pig kidney were negative As expected, allantoic and amniotic chick membrane suspensions neutralized the antibody

2 *Homologous and Heterologous HIN-Titres of Normal Allantoic Fluids*

Results of titrations are shown in Table 1 It is seen that the highest titres are given by the homologous allantoic fluids

TABLE 1

Homologous and Heterologous HIN Titres of Different Normal Allantoic Fluids

Antiserum against	Virus antigen	HIN titres of normal allantoic fluid from		
		chick	turkey	duck
Chick allantoic fluid normal	Chick allantoic Lee	150	< 2	20
Chick allantoic fluid PR 8 infected	Chick allantoic Lee	120	6	24
Turkey allantoic fluid PR 8 infected	Turkey allantoic Lee	< 2	20	12

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[illegible] E) The enzyme had been purified from crude [illegible] cles of adsorption to fowl red cells and elution [illegible] n added 0 1 per cent of $CaCl_2$ The acetate saline consisted of one volume of 0 2 N acetate buffer pH 6 2 and four volumes of normal saline The titre of the enzyme preparation was 640 with PR 8 as a test virus (1)

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activated in the boiling waterbath This enzyme was also tried only at 56° C for 2 hours without any HIN titre reduction The various treatments destroyed the normal inhibitor to PR 8

Because of these results it is assumed as a preliminary hypothesis that the HIN antigen is not a protein

It was found that also crude cholera filtrate failed to reduce the HIN titre of the allantoic fluid and this confirms that the filtrate could safely be used to destroy normal inhibitor prior to the examination of the HIN antigen

The effect of trypsin treatment on the sedimentation rate Allantoic fluid was dialysed against normal saline and subsequently incubated for 2 hours at 37° C pH 7 5 with an equal volume of 0 5 per cent trypsin which was a) active b) inactivated by heating at 56° C for ½ hour Aliquots of the two preparations were spun for 2 hours at either 25 50 80 110 or 140 000 × G After centrifugation the supernatants were treated with 0 3 per cent active trypsin at 37° C for 2 hours and subsequently heated at 56° C for 15 minutes When the unspun fluids and the supernatants were tested on their HIN titres preparation a showed the titre 112 unspun as well as after 140 000 × G while preparation b showed the titre 112 unspun 64 after 50 000 × G and 32 after 140 000 × G It is therefore assumed that the HIN antigen is bound to a protein although it may not itself be a protein

Treatment with periodate To one volume of allantoic fluid was added one volume of serial 2 fold dilutions of KIO_4 in distilled water The mixtures were kept at 37° C over night finally one volume of 5 per cent glucose was added The HIN titres were as follows (primary dilutions of the periodate) M 50 24 M 100 96 M 200 and less 192 It is seen that the antigen is moderately susceptible to the action of periodate and this may explain the inhibition examined in Section 9 a in the preceding paper (3)

Treatment with HCl To one volume of allantoic fluid were added 4 volumes of 1 N HCl the mixture kept in the boiling waterbath for 1 hour and subsequently neutralized with NaOH and phosphate buffer This treatment destroyed the HIN antigen

DISCUSSION

No other cross reactions than those between chick turkey and duck allantoic material were found in the HI test with host specific antibody Thus absorption with guinea pig kidney had no effect on the titre of this antibody In the complement fixation test on the other hand *Smith et al* found that the cross reactivity between influenza virus and host tissue extract was due to antigens of the Forssman type (4)

The preliminary examination of the chemistry of the HIN antigen in normal chick allantoic fluid showed that it was resistant to various proteolytic enzymes and therefore less likely to be a protein It may

TABLE 2

The Influence of the Age of the Chick Embryos on the HI and the HIN-Titre of the Normal Allantoic Fluid

Age of the embryos days	8	9	10	11	12	13	14	15
Number of eggs examined	2	10	10	10	10	10	10	10
HI titres	1	1	2	4	2	2	8	8
HIN titres	*24*	*32*	*48*	*48*	*56*	*56*	*128*	*128*

The homologous HIN titres of normal allantoic fluids from chick embryos of different ages were also examined When the fluids were tested against 5 haemagglutinating doses of chick allantoic PR-8 and 5 inhibiting doses of an antiserum against normal chick allantoic fluid, the titres shown in Table 2 were recorded These fluids had not been pretreated with cholera filtrate and their HI-titres are also shown in the table The amniotic fluids had lower HIN-titres than the corresponding allantoic ones Also the titres of the amniotic fluids increased when the embryos became older

3 *The Chemical Structure of the Normal Allantoic HIN Antigen*

Normal chick allantoic fluid, chick allantoic PR-8 and antiserum against normal chick allantoic fluid or chick allantoic Lee were employed The allantoic fluid underwent different treatments, and their effect on the HIN-antigen was titrated

Dialysis Attempts to dialyse the antigen through cellulose casings (Visking Corp) were negative This shows that the HIN-activity of the allantoic fluid is not due to a lowmolecular hapten The following experiment lead to the same conclusion

Precipitation with alcohol Allantoic fluid was dialysed against distilled water, freezedried and subsequently dissolved in 1/16 of the original volume of d w Ethylalcohol was added and the mixture left to stand over night at +4° C When the final concentration of alcohol was 90 per cent, all HIN-antigen was precipitated When the concentration was 45 per cent, very little antigen was precipitated

Heating The HIN-titre was not reduced when the allantoic fluid was kept for 1 hour in the boiling waterbath

Treatment with RDE One ml of allantoic fluid was treated with 4 ml of the RDE-preparation at 37° C over night followed by heating at 62° C for ½ hour This treatment did not reduce the HIN-titre

Treatment with proteolytic enzymes No reduction of the HIN titre was obtained when the allantoic fluid was treated at pH 7 5 with 0 5 per cent w/v concentrations of the following enzymes crystalline bovine pancreatic trypsin crystalline bacterial trypsin crystalline chymotrypsin, crude papain and ficin The treatments took place for 2 hours at 37° C, subsequently the enzymes were inactivated by heating for ½ hour at 56° C, except that the bacterial trypsin had to be in-

activated in the boiling waterbath This enzyme was also tried only at 56° C for 2 hours without any HIN titre reduction The various treatments destroyed the normal inhibitor to PR 8

Because of these results it is assumed as a preliminary hypothesis that the HIN antigen is not a protein

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however, be a carbohydrate attached to protein The same substance is probably part of (or absorbed to) the viral haemagglutinin

Frisch-Niggemeyer & Hoyle found that the influenza haemagglutinin is a protein which contains 4 2 per cent of carbohydrate, and no lipids or nucleic acids (2) *Zillig et al* arrived at an estimate of 10 per cent of carbohydrate in fowl plague haemagglutinin (5) They found that treatment with trypsin did not completely destroy the haemagglutinating and complement fixing power of the haemagglutinin, and mentioned that the carbohydrate might be of antigenic importance

SUMMARY

Haemagglutination inhibition neutralization (HIN) tests with purified allantoic influenza viruses, anti host sera and chick, turkey and duck normal allantoic fluids showed that the HIN-antigens of these fluids are related, but different from each other, and not present in chick embryo viscera or guinea pig kidney The concentration of the HIN-antigen in normal chick allantoic fluid increased when the embryos became older

The antigen could not be dialysed, and was precipitated by alcohol It was resistant to boiling, to tryptic enzymes, papain and ficin, and to RDE Before treatment with trypsin most of it was spun down at 140 000 × G, but nothing after treatment The antigen was moderately susceptible to treatment with periodate, and was destroyed when heated with dilute HCl It is tentatively assumed that the antigen is a high-molecular carbohydrate, which exists combined with a protein

REFERENCES

1 *Burnet F M & Stone J D* The receptor destroying enzyme of V cholerae ... Biol & Med Sc 25 227 233 1947

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BRIEF REPORTS

A MODIFIED METHOD FOR THE PREPARATION OF THE OSSIFIED SKELETON OF MOUSE FETUSES

By *Lars Jacobsen*

The past decade's increasing production and application of new and complicated pharmaceutical drugs—often having a wide range of effects on the mammalian organism—has brougt with it an added need for more thoroughgoing investigations into the substances' teratogenic effects This need has in the last few years been further accentuated by the thalidomide catastrophe

From the United Nations' report in 1962 and many recent works concerned with teratologic aspects (*Asling et al* 1955 *Degenhardt et al* 1959 *King et al* 1962) it appears that in mice and rats the anlage of the embryonic skeleton forms a system extremely sensitive to external influences It has therefore been felt to be of interest to make known a simple relatively quick preparation technique for the ossified skeleton of a mouse fetus immediately before birth The method is based on a modification of that made known by L B Russel (*Russell* 1956)

One day before the birth nearly the whole skeleton is ossified and in the remaining as yet unossified part nearly all bone anlage contain ossification centra The method can also be used on newborn mice up until 3–4 days of age

1 The fetuses are removed from the uterus and separated from the placenta, umbilical cord and membranes
2 *Exenteration* is carried out by means of two small pairs of curved tweezers (e g eye tweezers) The fetus is held with the one pair of pincers and the whole set of organs can be completely removed with the other pair through a horizontal cut in the abdomen Further from regio nuchae are removed 2 cushions of fat which from experience are an obstacle during further observation of columna cervicalis
3 *Fixation* with 70 per cent alcohol for 3 days The fixation and the subsequent procedures are most easily carried out by using Petriplates divided into 4 partitions
4 *Maceration* is undertaken with 1 per cent potassiumhydroxyd The best results are achieved after 5 days treatment with daily removal of the discharge At the same time loose bits of tissue and skin can be removed so long as damage to the columna and extremities is avoided
5 *Staining* is achieved with a solution of 0 70 g acid alizarinsulphonic sodium salt (alizarin S) in 1 l pure glycerine The process should take at least 72 hours
6 *Clearing* of the fetus takes place by replacing the coloured discharge with pure glycerine After 48 hours the glycerine is renewed and the preparation can thereafter be kept for at least two years

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References *Asling C W* *Nelson M M* *Wright H V* & *Evans H M* The Anatomical Record *121* 775 799 1955—*Degenhardt K H* & *Knoche E* The Canadian Medical Association Journal *80* 441 445 1959—*King C T G* & *Kendrick F J* The Lancet 7265 vol 2 1116 1962 *Russell L B* The Journal of Experimental Zoology, *131* 329 395 1956 *United Nations* Report of the United Nations Scientific Committee on the Effects of Atomic Radiation Supplement No 16 (A/5216) New York United Nations 1962 pp 442

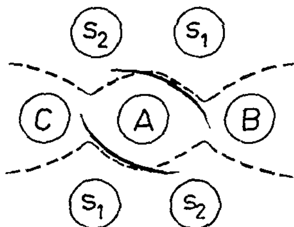
Received 12 III 63 from the University Institute For Human Genetics (Head Professor Tage Kemp M D) Department of Experimental Genetics and Cytology (J Schultz Larsen M D)

STAINING OF POLIOVIRUS ANTIGENS WITH THE FLUOROCHROME ACRIDINE ORANGE

By *Marianne Ohlson*

Staining with the fluorochrome acridine orange was applied to poliovirus antigens separated in agar gel double diffusion (micro scale) Under controlled conditions this stain is considered to be a specific nucleic acid stain A staining procedure adapted by *Mayor & Diwan* (1 2) for studies of purified poliovirus preparations was used The dye is to some extent attached to the agar If no fixation is used the agar fluoresces very faintly If stained after fixation the agar fluoresces in green providing a green background to the red RNA fluorescence

In native poliovirus preparations two separate antigens are encountered a heat sensitive (D) and a heat stable (C) antigen (3) Treatment at 56° C rapidly converts *the D antigen to C (3) In immunodiffusion D and C can easily be separated against* appropriate hyperimmune sera or human sera However the spontaneous C and the heated antigen cannot be distinguished from each other by immunological means as illustrated by the reactions of full identity in the fig



Figure

treated 30 min at 56° C containing only heated C S_1 guinea pig hyperimmune serum against native antigen containing anti D and anti C (spontaneous) S_2 guinea pig hyperimmune serum against heated antigen containing only anti C (heat)
——— *D antigen antibody precipitate showing red fluorescence*
C antigen antibody precipitate showing n fluorescence

The three kinds of polio virus precipitates containing D C or heated antigens were stained with 0 01 per cent *acridine orange at pH 4 0 To exclude the possible* influence of variations in staining between different agar plates the various antigens orescence
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fluores
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precipitate showed this red fluorescence Neither the spontaneous C nor the heated antigen did fluoresce in red *not even after fixation A faint green fluorescence* might however have been obscured by the green background
Identical results were obtained with all three types of poliovirus (type 1 Brun

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hilde, type 2 a wild strain isolated in Stockholm in 1954 Sthlm 5365, type 3 Saukett)
It is thus evident that the D antigen-antibody complex shows RNA fluorescence with acridine orange The attachment of antibody to the virus does not inhibit the attachment of the dye

' C precipitates supports the the C fraction (4) Possibly d in electron microscopy of

By heating the D antigen at 56° C its staining capacity is lost This could be explained in several ways

1 The nucleic acid has escaped through the damaged protein capsid
2 The denatured protein capsid is impermeable to the stain
3 The steric structure of nucleic acid is lost

This staining of poliovirus antigen antibody precipitates provides a good tool for identification of the D antigen

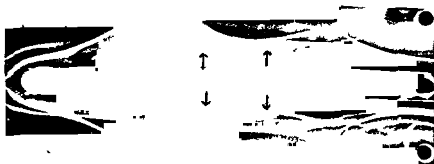
References 1 *Mayor H D & Diwan A R* Virology *14* 74–82 1961 —2 *Mayor H D* Progr med Virol *4* 70 86 1962 —3 *le Bouvier G L* Brit J Exp Pathol XL 452 463 1959 —4 *le Bouvier G L Schwerdt C F & Schaffer F L* Virology 4 590–593 1957 —5 *Horne R W & Nagington J* J Mol Biol *1* 333 338 1959

EXPERIMENTAL TRANSFORMATION OF THE GROUP SPECIFIC COMPONENTS (Gc) OF SERUM INTO A SINGLE ALPHA 1 GLOBULIN IMMUNOLOGICALLY IDENTICAL WITH THE Gc

By *B Nerstrom*

In addition to the normal Gc groups *Hirschfeld* described variant patterns showing abnormally fast migrating Gc precipitates (1)

During extensive family investigations performed at the present laboratory concerning the heredity of the Gc groups a similar pattern was encountered only once and the sample in question was old and apparently grossly contaminated By examination of a fresh sample from the same person a completely normal 1 1 pattern was demonstrated (2)



Immunoelectrophoretic pattern of serum from a hemolysed blood sample from a child displaying an asymmetrical Gc 2 1 precipitate and the new α₁ globulin precipitate above The serum reference presents a normal Gc 2 1 precipitate (below) By examination of a fresh serum sample from the child a normal Gc 2 1 precipitate was found

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However, during an investigation of the Gc groups in dry blood stains a distinct transformation of the Gc patterns was observed (3) The normal Gc precipitates were more or less weakened or had completely disappeared concurrently with the formation of a new precipitate in the α_1 globulin region immunologically identical to the Gc factors The transformation appeared to affect the 2 component earlier than the 1 component but the configuration and localization of the new α_1 precipitate was independent of the original Gc type of the sample Similar changes were found in some grossly hemolysed samples especially small samples of capillary blood from children (see figure) or samples which accidentally had been frozen soon after they had been withdrawn The abnormal pattern was not observed in serum samples which had been dried or frozen after separation from the cells

Thus a disintegration of fresh cells in the serum appeared to be a condition of the transformation which as a working hypothesis might be due to an enzymatic action Experimental results appeared to corroborate this assumption Fresh samples of whole blood were kept overnight at 20 degrees below zero and after thawing incubated at 37 degrees centigrade Already after 2 hours but more pronounced after 4 hours incubation all samples showed the characteristic patterns described above

In preliminary experiments using cell free serum and pure cell fractions it was shown that the transformation was produced by disintegrated leucocytes and thrombocytes whereas admixture of undamaged cells or disintegrated erythrocytes did not provoke the transformation Using disintegrated leucocytes in varying dilutions the whole range of transformations from a slight anodic extension of the normal Gc precipitate to a complete disappearance of the latter and the formation of a pronounced new α_1 precipitate was demonstrated

In further experiments a similar transformation has been produced by treating the serum with disintegrated yeast cells and with a commercial proteolytic enzyme (bacterial proteinase NOVO)

References 1 *Hirschfeld J* Progr Allergy *6* 155 186 1962 —2 *Nerstrom B* Acta genet (in press) —3 *Nerstrom B & Skafte Jensen J* Acta path et microbiol scandinav (in press)

INDEX

VOL 57 FASC 1–4 1963

SUPPLEMENTA